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<b>(54) Title: ANTI-IDIOTYPE ANTIBODIES TO HUMAN MELANOMA-ASSOCIATED PROTEOGLYCAN ANTIGEN</b>			
<b>(57) Abstract</b>			
<p>This application discloses a novel anti-idiotypic antibody, IMelpg2 and equivalents thereof, as well as, antibody fragments, peptides or antisera capable of reacting with at least one of the idiotypes of: (a) murine monoclonal antibody MEM136 and derivatives thereof; (b) a monoclonal antibody secreted by hybridomas from any species having the same immunological specificity as antibody MEM136; or (c) any polyclonal antibodies from any species having the same immunological specificity as antibody MEM136, wherein (a), (b), or (c) is capable of reacting with a specific determinant (epitope) of an MPG antigen are described, together with their preparation and use in the diagnosis, monitoring and treatment of tumors such as melanoma or other diseased cells that express the MPG epitope recognized by antibody MEM136.</p>			

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DESCRIPTIONAnti-Idiotype Antibodies to Human  
Melanoma-Associated Proteoglycan AntigenReference to Related Application

This application is a continuation-in-part of U.S.  
Ser. No. 07/431,426, filed November 3, 1989

Field of the Invention

5       The present invention relates generally to the development of antibodies that are specifically designed to enhance immunological activity by the host against a tumor. More particularly, the present invention relates to antibody reagents that are directed to antigens  
10      expressed on the melanoma cells and are useful in the treatment of patients suffering from melanoma. Such antigens include, the High Molecular Weight-Melanoma Associated Antigen ("HMW-MAA"), also known as the human melanoma-associated proteoglycan ("MPG"). MEM136 is a  
15      monoclonal antibody that recognizes an epitope of undefined structure on the MPG antigen.

Accordingly, this invention is in the field of anti-idiotypic antibodies to the anti-MPG antibody MEM136 and its immunological equivalents. The anti-idiotype antibody  
20      IMelpg2 (also known as "IM32") to antibody MEM136 has been discovered and evaluated in animal model systems.

This invention also is in the field of diagnosis, monitoring and treatment of tumors, such as melanoma or other diseased cells that express the MPG epitope recognized by antibody MEM136, by the administration and application of such anti-idiotypic antibodies.

Background of the InventionIntroduction

Antigens are substances that can induce an immune response, for example, a "humoral response" or B-cell lymphocyte response that results in antibody production. Another typical response is the stimulation of T-cells, viz., a "cellular response." (T-cells are another type of lymphocyte that have undergone differentiation in the thymus gland.) T-cells are capable of destroying abnormal cells such as those infected by certain types of viruses or other pathogens. T-cells also are involved in antibody production by B-cells. Accordingly, an antigen can react either with antibodies or with receptors on the T-cells that it stimulates. Antibodies and T-cell receptors are specific for the inducing antigen.

Antibodies and T-cell receptor molecules possess variable regions that are responsible for specific antigenic recognition. The region of the antigen that actually binds to the antibody or T-cell receptor is termed the antigenic determinant or "epitope." Similarly, the variable regions of antibodies and T-cell receptors also contain determinants, or "idiotypes" that are immunogenic and are capable of initiating an anti-antibody antibody response, i.e., an anti-idiotype ("anti-id") immune response.

More particularly, idiotypes are associated with the variable regions of antibody and T-cell receptors. These variable regions confer antibody and T-cell receptor specificity for antigens. Idiotypes are immune system markers on antibodies and T-cell receptors. An idiotype is immunologically defined by reactivity with more than one anti-idiotypic antibody that recognizes an idiotypic determinant or idiotope within a given idiotype; i.e., an idiotype is made up of a collection of idiotopes. Idiotopes are best defined by their binding to monoclonal anti-idiotypic antibodies. It also should be noted that

idiotopes are distinct from isotypic (immunoglobulin class-specific), xenotypic (species specific) and allo-typic (certain population sub-group specific) determinants.

5        Each antibody and T-cell receptor has at least one paratope that is the binding site for an antigen determinant (the epitope). A paratope may serve as an idiotope, i.e., the paratope may stimulate an anti-idiotypic response in which, like the original antigen, an anti-  
10      anti-idiotopic antibody bind to an epitope within the paratope. A subset of anti-paratope anti-idiotype ("anti-id") antibodies may mimic the immunologic properties of the original antigen and are known as "Ab2 betas" or "internal images" antibodies. In addition to the anti-  
15      paratopic anti-ids that mimic the original antigen, other anti-id antibodies define antibody and T-cell receptor idiotypes that also participate in the regulation of immune responses. These idiotypes are termed regulatory idiotypes and they are not necessarily "internal images"  
20      of the original antigen. For a general discussion of these background principles, see Burdette, S. and Schwartz, R., New Eng. J. of Med. 317:219 (1987).

#### The Idiotypic Network

In any given species or individual, the number of  
25      different antibodies and T-cell receptors is very large ( $>10^7$ ). Each antibody and T-cell receptor expresses several idiotypes that are linked through a regulatory network to complementary anti-idiotypes. Jerne, N., Ann. Immunol. 1256:373 (1974), proposed that the immune system  
30      is regulated by an interactive network composed of idiotypes and anti-idiotypes. There is a large body of experimental evidence indicating that the function of the idiotypic network is to maintain the homeostasis of the immune system and to regulate immune responses. As idiotypes are expressed by B-cells and T-cells, the idiotypic network encompasses both humoral (antibody) and cellular

(T-cell mediated) immune responses. Specific immune responses may be regulated by specific anti-idiotypic antibodies either on a one to one basis, or in groups through shared idiotypes. (See, Goldberg., et al., J. 5 Exp. Med. 158:515 (1983)).

#### Anti-idiotype Therapy

Recently, the use of "internal image" anti-idiotypes in therapy has been suggested and demonstrated in several experimental systems (EPO App. No. 848105169, filed 10 October 15, 1984, Pub. No. 0141783; Nisonoff, A., and Lamoyi E., Clin. Immunol. Immunopatholo. 21:397 (1981); Kennedy, et al., Biotechniques 3:4040 (1985)). These internal image anti-idiotypes have been used as surrogates for antigens in generating specific immune responses 15 against viral, bacterial and parasitic infections and cancers (Herlyn, et al., Science 232:100 (1986); Raychaudhuri, et al., J. Immunol. 137:1743 (1986)).

Currently, internal image polyclonal and monoclonal anti-idiotype antibodies are being used for active immuno- 20 therapy in animals and man. The methods for generating such anti-idiotype antibodies are well known to those of skill in the art. Briefly, the process of generating such internal image anti-idiotypes is as follows: (1) an antibody is made against an antigen or an infectious agent 25 (this antibody is called an "Ab1"); and, (2) anti-idiotypic antibodies ("Ab2s") are made against these Ab1s. These anti-ids (Ab2s) are screened for the expression of 30 idiotypes that mimic the immunological properties of the initial antigen (such as that expressed by a tumor or infectious agent). This screening consists of inhibiting the Ab1-Ab2 interaction by the original antigen and the biological testing of the anti-idiotypes (Ab2) as a surrogate antigen in vivo. This testing is done in animals and 35 has been demonstrated previously for internal image anti-idiotypes representing tumor antigens and infectious agents (Kennedy, et al., Biotechniques 3:4040 (1985);

Herlyn, et al., Science 232:100 (1986); Raychaudhuri, et al., J. Immunol. 137:1743 (1986).

Prior art efforts to generate useful active tumor immunotherapies, however, have met with unpredictable success. One may postulate several reasons for the limited success and unpredictable results seen in the prior art. First, many tumor antigens are difficult to isolate. Tumor antigens often are not present in significant concentration in tumor masses or on individual cultured tumor cells. Secondly, tumor antigens often are poorly immunogenic. The poor immunogenicity may be a result of the presence of other constituents of tumor cells that suppress immunologic reactivity, or because the antigens are not uniquely expressed by tumor cells. Generally, antigens that the immune system recognizes as "self" will not provoke a significant immune response. Thus, the use in tumor immunotherapy of anti-idiotype antibodies as tumor antigen surrogates has a number of advantages, including the ability to manufacture such antibodies in large quantities and the increased immunogenicity of such antibodies as compared to natural antigens.

Moreover, prior art efforts to generate useful internal image anti-idiotypes also have met with unpredictable success. With reference to the idiotypic network, many anti-idiotype antibodies, for reasons that are unclear, simply are unable to induce efficacious immune responses in vivo despite preliminary analyses indicating that they mimic the properties of the original antigen. Even anti-idiotype antibodies that demonstrate the proper specificity in vitro, or even partially in vivo, often are unable to stimulate the immune network to generate a therapeutically effective immune response. Presumably, these ineffective internal image Ab2s do not induce the appropriate regulatory circuits of the immune network of idiotype/anti-idiotype interactions to elicit effective immunity. In short, discovery of therapeutic-

ally useful anti-idiotype antibodies is as much art as science. Against this backdrop of unpredictability and uncertainty, the novel anti-idiotype antibodies herein described have been successfully tested to produce anti-melanoma tumor response in animals.

5 Melanoma Antigen Targets

One appropriate target to develop immunotherapeutic approaches to melanoma is the melanoma-associated chondroitin sulfate proteoglycan ("MPG") antigen, also referred 10 to as the high molecular weight melanoma-associated antigen ("HMW-MAA"). Applicant's results show that attempts to induce cellular and humoral responses against MPG may result in intervention of tumor growth. The MPG consists 15 of two noncovalently associated glycopolypeptides. One has an apparent molecular weight of 280Kd, and the other has an apparent molecular weight greater than 440Kd. MPG is synthesized and expressed by human melanoma cells 20 (Spiro, et al., J. Biol. Chem. 264:1779 (1989); Esko, et al., Science 241:1092 (1988)). Proteoglycans are glycoproteins with glycosaminoglycan (GAG) polysaccharide chains covalently attached to the serine amino acid residue in their core. The MPG core protein is initially 25 translated as a precursor with a molecular mass of 240Kd with asparagine-N-linked oligosaccharides of the high mannose type. Trimming and subsequent processing of the N-linked oligosaccharide to the "complex" form results in the conversion of the 240Kd component to a 250Kd form. The addition of chondroitin sulfate GAG side chains to the 30 250Kd core protein converts it to a high molecular weight (>450Kd) proteoglycan form. Both 250Kd and >450Kd forms are expressed on the melanoma cell surface (Spiro, et al., J. Biol. Chem. 264:1779 (1989)). MPG plays a key role in determining the fate of tumor growth and metastasis 35 (Herlyn, et al., Ann. Rev. Immunol. 6:283 (1988); Robertson, et al., Cancer Res. 49:1816 (1989)). Cell surface protein molecules are involved in interactions

with adjacent cells and form complex footpads that make contact with substratum (Herlyn, et al., Ann. Rev. Immunol. 6:283 (1988)). Antibodies to MPG have been reported to block chemotactic and chemokinetic motility of 5 cells (Herlyn, et al., Ann. Rev. Immunol. 6:283 (1988); de Vries, et al., Int. J. Cancer 38:465 (1986)). The anti-MPG antibodies cause the reduction of melanoma colony formation in soft agar (Harper, J. Natl. Cancer Inst. 71:259 (1983)).

10 Against this background of information and uncertain results, Applicant has discovered anti-MEM136 antibodies that are clinical candidates for use in tumor immunotherapy.

#### Summary of the Invention

15 In its broadest aspect, the present invention is directed to a novel anti-idiotypic antibody, antibody fragment, peptide, or antisera capable of reacting with at least one of the idiotypes: (a) murine monoclonal antibody MEM136 and derivatives thereof; (b) a monoclonal 20 antibody having the same immunological specificity as antibody MEM136; or (c) polyclonal antibodies having the same immunological specificity as antibody MEM136, wherein (a), (b), or (c) is capable of reacting with a specific determinant (epitope) of a MPG antigen.

25 Additional aspects of the present invention include methods for using the novel anti-idiotype antibodies in active and passive immunotherapy, as well as the diagnosis, monitoring, and treatment of melanoma and other disease states associated with the epitope recognized by 30 the MEM136 antibody.

#### Brief Description of the Drawings

FIGURE 1 depicts the fine specificity of Ab1s, in particular MEM136.

35 FIGURE 2 illustrates that Ab2 binding is MEM136- $F(ab')_2$ -specific.

FIGURES 3A & 3B illustrate that the Ab2s are binding site idiotope-specific.

FIGURE 4 shows that MEM136 was the only antibody that could effectively inhibit binding of  $^{125}\text{I}$ -Ab2 to MEM136.

5 FIGURE 5 shows the anti-MEM136 Ab2s could be grouped into two categories based on their inhibitory activity.

FIGURE 6 depicts isoelectric focusing of anti-MEM136 Ab2s.

FIGURE 7A-H depict the inhibition by rabbit anti-  
10 anti-idiotypic antisera of the binding of  $^{125}\text{I}$ -Ab2s to  
MEM136.

FIGURE 8 shows idiotope localization of anti-MEM136 Ab2 antibodies.

FIGURE 9 depicts the Ab3/Ab1' response in rabbits  
15 immunized with the panel of Ab2s.

FIGURE 10 shows the Ab1' response in sera of rabbits immunized with IMelpg2 or IM06 in MDP-A as determined by IIF analysis.

FIGURE 11 depicts the Ab1' response in sera of  
20 rabbits immunized with IMelpg2 in MDP-A as determined by IIF analysis.

FIGURE 12 shows the total idiotype (Ab3) response in sera of rabbits immunized with IMelpg2 or IM06 with MDP-A.

FIGURE 13 depicts the Ab1' response in sera of  
25 rabbits immunized with IMelpg2 or IM06 in MDP-A.

FIGURE 14 depicts the total idiotype (Ab3) response in sera of mice immunized with IMelpg2 or IM06 with MDP-A.

FIGURE 15 depicts the Ab1' response in sera of a representative mouse immunized with IMelpg2 or IM06 in  
30 MDP-A as assayed by IIF

FIGURE 16 depicts the Ab1' response in sera of mice immunized with IMelpg2 with MDP-A as assayed by inhibition of MEM136 binding to Colo38.

FIGURE 17 shows the IIF analysis of Ab1' anti-  
35 melanoma antibody in affinity purified rabbit Ab3.

FIGURE 18 depicts the IIF analysis of IMelpg2-Ab3 on Colo38 (MPG<sup>+</sup>) cells before and after absorption on Colo38 and NMB7 cells.

FIGURE 19 shows the inhibition of binding of <sup>125</sup>I-MEM136 to Colo38 cells by affinity purified rabbit Ab3s obtained from rabbits immunized with IMelpg2 or IM06 in MDP-A.

FIGURE 20 A and B shows the inhibition of binding of <sup>125</sup>I-IMelpg2-Ab3 to IMelpg2 by various concentrations of unlabeled IMelpg2-Ab3 and IM06-Ab3, (A) and the inhibition of <sup>125</sup>I-IMelpg2-Ab3 binding to IMelpg2 by different dilutions of sera from mice immunized with IMelpg2 or IM06 (B).

FIGURE 21 depicts the Ab1' response profile in the IMelpg2-Ab3 population absorbed on IM06.

FIGURE 22 shows the idiotype response of IMelpg2-Ab3, before and after absorption on IM06-sepharose using inhibition of binding of <sup>125</sup>I-MEM136 to IMelpg2.

FIGURE 23 shows the inhibition of binding of <sup>125</sup>I-MEM136 to Colo38 by IMelpg2-Ab3, before and after absorption on IM06-sepharose column.

#### Detailed Description of the Preferred Embodiments

The following terms, as used in the disclosure and claims, are defined as follows:

"passive immunotherapy" -- passive transfer of antibody to a patient or animal.

"active immunotherapy" -- the induction of antibody and/or T-cell responses in a patient or animal.

"suppression" -- the inhibition of an immune response or the inhibition of the expression of an idiotypic response.

"stimulation" -- the induction of an immune response or the induction of the expression of an idiotypic response.

"tumor regression": an overall 30% or greater reduction of tumor mass.

All of the references cited in this disclosure are hereby incorporated by reference.

As previously discussed, the potential for utilizing anti-idiotypic (anti-Id) antibodies to induce active anti-tumor responses in patients with neoplastic disease is enormous. Applicant has explored the feasibility of using anti-Id antibodies (Ab2s) as immunoactivators to potentiate tumor-specific B and T cell responses in melanoma patients. Melanoma-associated chondroitin sulfate proteoglycan antigen (MPG) is synthesized and expressed by human melanoma cells. Proteoglycans are glycoproteins with glycosaminoglycan polysaccharide chains covalently attached to the serine residues in their core. The various biological properties of MPG in melanoma have been previously described. Briefly, MPG is involved in chemotactic and chemokinetic cell motility, and anti-MPG antibodies cause the reduction of melanoma colony formation in soft agar.

MEM136 is a monoclonal antibody ("MoAb") which recognizes an epitope of undefined structure on the MPG molecule. When tested in an immunohistology study, MEM136 reacted to 80% of melanoma tissues, with minimal cross-reactivity to normal tissues. Therefore, an MPG epitope detected by MEM136 can serve as a potential tumor-associated antigen. Accordingly, as described below, Applicant has (i) generated and characterized several site-specific anti-id antibodies; (ii) analyzed V-gene usage of select Ab2s by biochemical and immunochemical means, and (iii) screened the Ab2's for the ability to induce an anti-tumor response in small animals.

Example 1Development and Characterization of the Anti-idiotypic Monoclonal Antibody (IMelpg2) Directed to the Monoclonal Antibody MEM136 Directed Against MPG Antigen5 Materials and Methods

The human melanoma cell line Colo38, obtained from Dr. Soldano Ferrone at New York Medical College, was grown in RPMI 1640 medium supplemented with 10% calf serum, 1% glutamine and 10 mg/ml of gentamycin sulfate. The anti-Id 10 antibodies developed in connection with the present invention were produced by hybridomas generated from a fusion of splenocytes from A/J mice immunized with MoAb MEM136 and murine myeloma cell line SP2/0.

Monoclonal Antibodies

15 The MoAb MEM136 ( $\gamma_1, \kappa$ ) (available from Hybritech, Inc., San Diego, CA) was purified on a protein A-Sepharose 4B column. MOPC21 ( $\gamma_1, \kappa$ ) anti-idiotype antibody ("MOPC21") (available from Bionetics Lab Products, Charleston, SC) was used as an isotype-matched control 20 for MEM136.

Preparation of F(ab')<sub>2</sub> Fragments

The concentration of MEM136 antibody solution in phosphate-buffered saline ("PBS") was adjusted to 1-2 mg/ml and the solution was dialyzed against 0.1 M of 25 citrate buffer, pH 3.5. Pepsin was then added to give a final concentration of 25  $\mu$ g/ml. The solution was incubated for 8 hours at 37°C. Thereafter, the reaction was stopped by increasing the pH to 8.0 by adding high salt 30 protein A buffer. After cycling over protein A, the flowthrough that contained the F(ab')<sub>2</sub> portion of the molecule was collected and dialyzed extensively against PBS. The purity of the preparation was checked by examining its reactivity against heavy and light chain-specific antibodies in an ELISA.

Generation of Anti-MEM136 Idiotype Hybridomas

A/J mice were immunized three times with 100 µg of MEM136 coupled to keyhole limpet hemocyanin ("KLH") in incomplete Freund's adjuvant ("IFA") i.p. over a period of 5 two months. Two weeks after the last injection, mice were boosted intravenous ("i.v.") with MEM136 antibody in PBS. Four days after this inoculation, the fusion was performed according to the methods described in Raychaudhuri, *et al.*, *J. Immunol.*, 137: 1743-1749, 1986, with SP2/0 as a 10 fusion partner and 50% polyethylene glycol, m.w. 1500. The hybrids were selected with HAT media.

Selection of Anti-MEM136 Idiotype Antibody (Ab2)

Initial screening of the hybridomas was carried out by ELISA in a binding assay with F(ab')<sub>2</sub> fragments of 15 MEM136 and MOPC21. Wells containing antibody that bound to the MEM136 F(ab')<sub>2</sub> fragment but not to the MOPC21 F(ab')<sub>2</sub> fragment were selected and additionally tested. The monoclonal anti-idiotope antibodies IM04 ( $\gamma_1, \kappa$ ), IM06 ( $\gamma_1, \kappa$ ), IM08 ( $\gamma_1, \kappa$ ), IM09 ( $\gamma_1, \kappa$ ), IM13 ( $\gamma_1, \kappa$ ), IM20 ( $\gamma_1, \kappa$ ), IM30 ( $\gamma_2b, \kappa$ ) and IMelpg2 ( $\gamma_1, \kappa$ ) were selected for 20 further analysis.

Radioimmunoassay ("RIA")

Additional characterization of the Ab2s was done by RIA. Rabbit anti-mouse IgG was purchased from Southern Biotechnology Associates, Inc., Birmingham, AL, and was 25 radioiodinated as described in Raychaudhuri, *et al.*; *J. Immunol.*, 137: 1743-1749 (1986). A binding assay was performed as described in Raychaudhuri, *et al.*; *J. Immunol.*, 137: 1743-1749 (1986). The F(ab')<sub>2</sub> fragments of 30 either MEM136 or MOPC21 were added to PVC plates at a concentration of 100 to 200 ng/well. After overnight incubation at 4°C, the plates were blocked with 1% bovine serum albumin ("BSA") in PBS. Thereafter, varying dilutions of Ab2 culture supernatant were incubated at room 35 temperature for 4 hours. The plates were developed using

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10,000 counts of goat anti-mouse Ig in 100  $\mu$ l of PBS containing 1% BSA. The binding site specificity of Ab2s was determined by incubating Colo38 cells with  $^{125}$ I-MEM136 in the presence of varying concentrations of the Ab2s for 5 18 hours at 4°C. Thereafter, plates were washed and the counts bound to Colo38 were determined in a gamma counter.

Immunization of Rabbits

Adult New Zealand White ("NZW") rabbits (two per group) were immunized subcutaneously at different sites 10 with 500  $\mu$ g of unconjugated IM04 through IM30, and IMelpg2 in Syntex Adjuvant Formulation("SAF")-m adjuvant containing 250  $\mu$ g of threonyl muramyl dipetide (threonyl "MDP") per rabbit. Rabbits were boosted with the same antigen concentration every 2 weeks and bled 7 and 14 days after 15 each immunization. Each rabbit was immunized a maximum of four times. Control rabbits were immunized with 500  $\mu$ g of MOPC21 or KLH alone.

Analysis of Anti-Anti-Idiotypic (Ab3) Response

The idiotypic responses induced by IM04, IM06, IM08, 20 IM09, IM13, IM20, IM30 and IMelpg2 were analyzed by RIA. Briefly, 100 ng of MEM136-F(ab')<sub>2</sub> fragment was coated onto microtiter plates for two hours at room temperature. The plates were washed with PBS and then incubated with 1% bovine serum albumin (BSA) in PBS for one hour. Serially 25 diluted sera from different experimental and control groups were incubated on the wells in the presence of 20,000 cpm of  $^{125}$ I-Ab2s for 18 hours. The plates were thoroughly washed and the radioactivity in each well was counted in a gamma counter. The results are expressed as 30 percentages of inhibition of  $^{125}$ I-Ab2 binding to MEM136.

Isoelectric Focusing of Ab2s

Isoelectric focusing was performed using a pH 3-10 isoelectric focusing gel (Novex, Encinitas, CA). After fixing, the gel was destained with 10% acetic acid and 25%

ethanol and then stained with the same solution containing 0.1% Coomassie Blue. pI standards were included to determine pH values (Pharmacia LKB Biotechnology, Piscataway, NJ).

5 Idiotope Localization of Ab2 Antibodies

Immunoblotting assays were performed to determine whether or not the epitopes recognized by the anti-idiotypic antibodies were detected on isolated heavy or light chains of MEM136. Monoclonal antibody MEM136 was 10 reduced with 5% 2-mercaptoethanol and its heavy and light chains separated in a 10% polyacrylamide-sodium dodecyl sulfate gel and transferred onto nitrocellulose using a Transphor blotting apparatus (Hoefer, San Francisco, CA). As a positive control, unreduced MEM136 was run on the 15 same gel. Control nitrocellulose strips were stained with Ponceau 5 Stain (Sigma Chemical Co., St. Louis, MO). Blots to be reacted with monoclonal anti-idiotypes were first blocked for 2 hours with BSA (3% in Tris-buffered saline). These blots were then incubated for 2 hours with 20  $^{125}$ I-labeled monoclonal anti-idiotype antibodies ( $1 \times 10^5$  cpm/ml in blocking buffer). After washing several times with Tris-buffered saline, the blots were dried and analyzed by autoradiography.

Fine Specificity of Monoclonal Anti-MPG Antibodies

25 The binding of  $^{125}$ I-labeled MEM136 to the melanoma cell line Colo38 was examined in the presence of unlabeled antibodies MEM136, 225, 155, 997, 998, 999 and UPC10, an unrelated isotype-matched control antibody. Antibodies 225, 997, 998, and 999 were obtained from Dr. Soldano 30 Ferrone, New York Medical College and the 155 antibody was obtained from Dr. J. Harper, Scripps Clinic and Research Foundation, La Jolla, CA. A human melanoma cell line, Colo38, was grown in each well of 96-well plates. After fixation and blocking, the binding of  $^{125}$ I-MEM136 to Colo38 35 was determined in the presence of different concentrations

of monoclonal anti-MPG antibodies 155, 225, 997, 998, and 999. As shown in FIGURE 1, unlabeled MEM136 was the only antibody that was able to efficiently inhibit the binding of  $^{125}\text{I}$ -MEM136 to Colo38. Dose ranges of 8-16 ng of this 5 antibody could inhibit the binding of  $^{125}\text{I}$ -MEM136 by 50%, while only 22-25% inhibition could be achieved with 225.28 at a concentration range of 256-512 ng, and no inhibition could be achieved with 155, 997, 998 and 999 at this 10 concentration. From this data, Applicant concludes that MEM136, 225.28, 155, 997, 998 and 999 detect distinct epitopes on the MPG molecule. Small amounts of inhibition seen with 225.28 could be due to steric hindrance contributed by high concentrations of 225.28 in the 15 reaction mixture.

15 Monoclonal Ab2s Recognize an Idiotope on MEM136

The hybridization of splenocytes from A/J mice immunized with MEM136 ( $\gamma 1, \kappa$ ) with SP2/0 cells yielded many hybridomas. The hybridomas IM04, IM06, IM08, IM09, IM13, IM20, IM30 and IMelpg2, which showed the highest binding 20 activity with MEM136 and no binding with the isotype-matched control antibody MOPC21, were subcloned and ascites were produced in BALB/c mice. Various concentrations of different Ab2s (IM04 through IM30 and IMelpg2) were incubated with  $\text{F}(\text{ab}')_2$  fragments of MEM136 coated onto 25 each well of 96-well plates. After one hour of incubation, the plates were washed and developed with peroxidase-conjugated goat anti-mouse IgG. As control, various concentrations of an unrelated Ab2, IC5, were added. FIGURE 2 shows the binding of different concentrations of these purified Ab2s to MEM136 - $\text{F}(\text{ab})_2$  fragment. 30

To determine the site specificity of these anti-id antibodies, the binding of  $^{125}\text{I}$ -labeled MEM136 to Colo38 cells was determined in the presence of different Ab2s. The Ab2s could be divided into two distinct categories 35 according to their inhibition capabilities. Group I, consisting of IM04, 08, 30 and IMelpg2, required <2 ng/well

to inhibit the binding of  $^{125}\text{I}$ -labeled MoAb MEM136 to Colo38 by 50%. Group II, consisting of IM06, 09, 13 and 20, required a concentration of 50 ng/well to achieve a 45-70% inhibition of binding. This binding inhibition by Ab2s in groups I and II appears to be specific since Ab2s elicited by an unrelated MoAb did not affect the binding of MEM136 to Colo38. Graph 3A shows the inhibition of  $^{125}\text{I}$ -MEM136 binding to melanoma cells in the presence of different concentrations of IM04, IM08, IM30 and IMelpg2. Graph 3B demonstrates the same study in the presence of different concentrations of IM06, IM09, IM13 and IM20. A known concentration of IC5 was used as a control in both studies. Ten ng of IM04, IM08, IM30 and IMelpg2 inhibited the binding of  $^{125}\text{I}$ -MEM136 to tumor cells by 80-95%. With IM06, IM09, IM13 and IM20, 80% inhibition of binding was not achieved at any concentration. These results suggest that the idiotopes recognized by some Ab2s (group I) are closely related to the antigen combining site of MoAb MEM136. In the following experiments, IMelpg2 and IM06 were chosen to represent group 1 and group 2, respectively, for further characterization of these Ab2s.

Ab2 Recognizes a Private Idiotypic Determinant on MEM136

Idiotypic relationships among monoclonal anti-tumor antibodies MEM136, 155, 225.28, 996, 997 and 998 with IM06 and IMelpg2 were analyzed in a competition assay, where the binding of IM06 and IMelpg2 to MEM136 was determined in the presence of different Ab1s. As shown in FIGURES 4A & 4B, the MEM136 idiotope recognized by IMelpg2 may share determinants with 225 and 155 distantly(4A); and that the MEM136 idiotope recognized by IM06 does not share determinants with other Ab1s(4B). Microtiter plates were coated with MEM136 and incubated with  $^{125}\text{I}$ -IMelpg2 (4A) or  $^{125}\text{I}$ -IM06 (4B) overnight in the presence of different concentrations of MEM136, 155, 225, 997, 998 and 999. Thus, 100% inhibition of  $^{125}\text{I}$ -IMelpg2 binding to MEM136 could be achieved with 16 ng of MEM136, while 50% inhibition by

antibody 155 required 400-500 ng/well. Similarly, 225.28 inhibited the binding of  $^{125}\text{I}$ -IMelpg2 to MEM136 by only 15-20% at a concentration of 250-500 ng/well. On the other hand, the inhibition of  $^{125}\text{I}$ -IM06 binding to MEM136 could 5 be attained only with MEM136. These data confirm Applicant's premise that the Ab2s recognize an idiotope that may be present predominantly on immunizing MEM136.

Ab2s may Share a Determinant but Differ in their Fine Specificity

10 The spatial relationship of idiotypes recognized by IM04, 06, 08, 09, 13, 20, 30 and IMelpg2 was analyzed in a competition assay, where the binding of  $^{125}\text{I}$ -labeled IM06 or IMelpg2 to MEM136 was determined in the presence of different concentrations of cold Ab2s. As shown in FIGURE 15 5A & 5B, the differences in fine specificity of the Ab2s. Each well of polyvinyl plates was coated with 50 ng of MEM136 in PBS and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS for 1 hour and the inhibition of  $^{125}\text{I}$ -IMelpg2 (5A) and  $^{125}\text{I}$ -IM06 (5B) binding to MEM136 was 20 studied in the presence of different concentrations of various Ab2s. 5A: at 12 ng of different Ab2s, a range of inhibition of  $^{125}\text{I}$ -IMelpg2 binding to MEM136 of 0-50% was observed. 5B: at 12 ng of different Ab2s, a range of inhibition of  $^{125}\text{I}$ -IM06 binding to MEM136 of 16-80% was 25 observed. When iodinated IMelpg2 was used as a ligand, 80-95% inhibition of  $^{125}\text{I}$ -IMelpg2 binding to MEM136 was achieved with 50 ng/well of IM04, 08, 30 and IMelpg2. At the same concentration, IM06, 09, 13 and 20 exhibited only 60-75% inhibition of  $^{125}\text{I}$ -IMelpg2 binding to MEM136. When 30 IM06 was used as a radioligand, 50% inhibition of binding of  $^{125}\text{I}$ -IM06 to MEM136 was achieved with IM04, 06, 08, 09 and 13 at a concentration range of 12-16 ng/well. In the same inhibition assay, 50% inhibition of binding was achieved at a concentration range of 22-35 ng/well with 35 IM20, 30 and IMelpg2. These results suggest that all Ab2s inhibited the binding of  $^{125}\text{I}$ -IM06 or IMelpg2 to MEM136 in

a dose-dependent fashion. It appears, therefore, that the idiotope(s) recognized by these Ab2s are spatially linked or overlap with each other.

Analysis of the Variable Region of the Anti-Id MoAbs

5        Immunochemical analysis was performed to determine the variability of the V-gene usage of the Ab2s. All eight Ab2s focused between pH 6.5-7.6 and comprised between 2-4 components. As shown in FIGURE 6, the PIs of the monoclonal antibody Ab2s were determined using a pH 3-  
10 10 isoelectric focusing gel. Lane 1, IM04; lane 2, IM06;  
15 lane 3, IM08; lane 4, IM09; lane 5, IM13; lane 6, IM20;  
lane 7, IM30; and lane 8, IMelpg2. The spectrototype of each Ab2 was different, suggesting a difference in the variable region polypeptide sequence and/or carbohydrate  
15 moiety (FIGURE 6).

Serological analysis was performed to corroborate the spectrototype analysis data. As mouse Ab2 immunized xenogenic antisera was analyzed, it is likely that an Ab2-induced idiotype response contributed by both framework  
20 and complementary determining region determinants was detected. Ab3 antisera elicited by IM04, IM06, IM08, IM09, IM13, IM20, IM30 and IMelpg2 were incubated with <sup>125</sup>I-IM04 (6A), <sup>125</sup>I-IM13 (6B), <sup>125</sup>I-IM30 (6C), <sup>125</sup>I-IM06 (6D), <sup>125</sup>I-IM20 (6E), <sup>125</sup>I-IM04 (6F), <sup>125</sup>I-IM08 (6G) and <sup>125</sup>I-  
25 IMelpg2 (6H) on MEM136-coated microtiter plates. After overnight incubation, plates were washed and bound radioactivity was measured in a  $\gamma$ -counter. As shown in FIGURE 7, each antiserum containing anti-anti-idiotype antibodies (Ab3) inhibited the binding of the corresponding anti-Id MoAb to a different extent. The Ab3s induced by IM30 and IMelpg2 inhibited the binding of <sup>125</sup>I-IM04, -IM06, -IM13, -IM20, -IM30 and -IMelpg2 to the MEM136-F(ab')<sub>2</sub> fragment most efficiently. The binding of <sup>125</sup>I-IM04 and -IM08 to MEM136 could not be competed by IM30 and  
30 IMelpg2 induced Ab3s as effectively as the Ab3s induced by IM04, 08 and 13. The hierarchy of inhibition of binding  
35 IMelpg2 induced Ab3s as effectively as the Ab3s induced by IM04, 08 and 13. The hierarchy of inhibition of binding

of  $^{125}\text{I}$  - IM 06, 09, 13, 20, 30 and IMelpg2 binding to MEM136 by Ab3 sera induced by different Ab2s can be ranked as follows; IM30 and IMelpg2 > IM06, 09, and 20 > IM04 and IM8. On the other hand when binding of  $^{125}\text{I}$ -IM04 and IM08 to MEM136 MoAb1 is considered, IM04, 08 and 13 induced Ab3 sera demonstrated highest inhibitory activity. These results suggest that the Ab2s can be grouped into at least three categories based on their immunogenicity and the ability to induce anti-anti-idiotypic antibodies. If the differential reactivity patterns of the antisera do not reflect variability in immune responses of immunized rabbits, the idiotypes expressed by the eight Ab2s differed in their immunogenicity.

Immunoblotting of MEM136 using Monoclonal Anti-Idiotypes

Idiotypes may be present on the isolated heavy or light chains of immunoglobulins or result from the association of both chains (See, FIGURE 8). Monoclonal antibody MEM136 was reduced with 2-mercaptoethanol and its heavy and light chains separated in a 10% polyacrylamide-sodium dodecyl sulfate gel and transferred onto nitrocellulose. As a positive control, unreduced MEM136 was run on the same gel. The blots were then incubated with  $^{125}\text{I}$ -Ab2 antibodies and analyzed by autoradiography. To determine the identities of the idiotypes for Applicant's monoclonal anti-idiotypes, MEM136 was reduced and its heavy and light chains were electrophoretically separated and transferred onto nitrocellulose. Of the nine anti-idiotype antibodies tested, none reacted with isolated heavy or light chains (FIGURE 8). As a positive control, when MEM136 was electrophoretically resolved and blotted under non-reducing conditions, all nine anti-idiotypic antibodies showed strong reactivity (FIGURE 8). While all nine anti-idiotypic antibodies were unreactive with isolated heavy or light chains, the possibility remains that an idiotope present on a single chain could have been destroyed by reduction with 2-mercaptoethanol. This

consequence seems unlikely, however, since single-chain idiotypes are usually unaffected by 2-mercaptoethanol.

The melanoma-associated proteoglycan molecules are believed to play many important roles in potentiating metastatic properties of melanoma. During the process of invasion and metastasis, it is necessary for tumor cells to traverse the extracellular matrix (ECM). The basement membranes within the ECM are composed mainly of collagen type IV meshed laminin, entactin, fibronectin and proteo-glycans. A substantial body of evidence exists to suggest a close correlation between extracellular membrane degradation and tumor cell invasion and metastasis. Therefore if tumor cells are prevented from binding to the ECM, metastasis may be inhibited. To this end, a humoral response can be induced against MPG to develop antibodies which will act as antagonists and interfere with the binding of melanoma cells to the basal lamina or the basement membrane.

An antibody response can be induced against a tumor antigen by the use of anti-idiotype antibodies made against a monoclonal or polyclonal antibody directed against epitope(s) present on the tumor-associated antigen. The advantages of such an approach have been stated elsewhere (Kennedy, *et al.*, Biotechniques 3:4040 (1985); Herlyn, *et al.*, Science 232:100 (1986); Raychaudhuri, *et al.*, J. Immunol. 137:1743 (1986)). Applicant has described the development and characterization of anti-idiotype antibodies generated against a monoclonal anti-human MPG antibody, MEM136. Applicant has selected monoclonal anti-idiotype antibodies IM04, 06, 08, 09, 13, 20, 30 and IMelpg2 based on their binding capability to immunizing MEM136 relative to an isotype matched control antibody. The antibodies IM04, 08, 30 and IMelpg2 (group 1) almost completely inhibited the binding of MEM136 to the human melanoma cell line Colo38 at a concentration of 10-20 ng/well. The antibodies IM06, 08, 13 and 20 (group 2), on the other hand, only inhibited the binding of

MEM136 to Colo38 by less than 70%, even at a concentration of 50 ng/well. Thus, the Ab2 antibodies in group 1 have a much higher idiotope site specificity than those in group 2. A more detailed characterization was performed  
5 on antibodies IMelpg2 and IM06 were considered as representative Ab2s from group 1 and group 2 respectively for detailed characterization of other Ab2s.

The spatial relationship of idiotopes recognized by the Ab2s was analyzed in a competition assay, where the  
10 binding of  $^{125}\text{I}$ -IMelpg2 and IM06 to MEM136 was determined in the presence of different Ab2s. Antibodies IM04, 08,  
30 and IMelpg2 (group 1) inhibited the binding of  $^{125}\text{I}$ -IMelpg2 to MEM136 by 100% at a concentration of 50 ng/well. At the same concentration, IM06, 09, 13 and 20  
15 inhibited IMelpg2 binding to MEM136 by only 60-75%. However, when  $^{125}\text{I}$ -IM06 was used as a ligand, there was no clear cut relationship between the percent inhibition of  
 $^{125}\text{I}$  IM06 binding to MEM136 and the site specificity of the  
20 Ab2s. These data indicate that the idiotope(s) recognized by the different Ab2s overlap.

Spectrotype analysis demonstrated differences in the isoelectric point ("PI") of the different Ab2s. Immunochemical analysis was corroborated with serological data by analyzing the ability of Ab2-induced antisera to  
25 inhibit the binding of  $^{125}\text{I}$  Ab2s (IM04 through IMelpg2) to MEM136. As demonstrated in FIGURE 6, the Ab2s were immunologically distinct. Based on the cross-reactivity study, the Ab2s were grouped into three categories. IM30 and IMelpg2 immune sera (group 1) induced an immunologically similar anti-anti-idiotype (Ab3) response and most effectively inhibited the binding of  $^{125}\text{I}$ -IM06, -09, -13,  
30 -20, -30 and IMelpg2 to MEM136. IM04 and 08 immune sera (group 2) only minimally inhibited the binding of the above  $^{125}\text{I}$ -Ab2s to MEM136. IM06, 09 and 20 immune sera (group 3) inhibited binding of  $^{125}\text{I}$ -IM06, -08, -09, -13,  
35 -20, -30 and IMelpg2 to MEM136 at an efficiency intermediate to groups 1 and 2. The binding of  $^{125}\text{I}$ -IM04 and

-08 to MEM136, on the other hand, was most inhibited by immune sera from IM04 and IM08, respectively, while IM30 and IMelpg2 immune sera inhibited the same binding minimally. This data indicates that though IM04, 06, 30 and 5 IMelpg2 are all site-related, they are immunologically distinct.

Finally, in an attempt to localize the idiotope on the Ab1, MEM136 (Ab1) was run on SDS-PAGE gels under reducing and non-reducing conditions, blotted onto nitrocellulose and subsequently probed with <sup>125</sup>I-Ab2s. In all 10 cases the idiotope was located only on the native molecule.

To summarize, Applicant's data indicate that a library of both site-related and site-unrelated Ab2s has 15 been generated which are immunologically distinct. The next step in selecting an therapeutic candidate involves determining which of these anti-idiotype antibodies is best able to induce a tumor-specific humoral response.

Example 2

20 Assay Evaluation of IM06 and IMelpg2

Antibodies raised against melanoma proteoglycan epitopes have been shown to inhibit cell adhesion reactions and matrix driven chemotaxis. This leads directly to the consideration of their potential ability to interfere with 25 melanoma:matrix interactions required for malignant tumor cell invasion and metastasis. Preliminary experiments on invasion of matrices can be conveniently and relatively cheaply carried out in culture. Tissue culture experiments are also useful in defining the mechanisms of any 30 observed inhibition of cellular invasion. Increased cell surface expression of melanoma proteoglycans in cells cultured from invasive stages of melanoma, the shedding and incorporation of this material into the extracellular matrix and the demonstration of similar material in the 35 matrix surrounding endothelial cells in early invasive stages of neovascularization are all observations which

support the link of these proteoglycan epitopes with cellular invasion. The antibodies under consideration could block cell:matrix interactions in at least two ways: a) by binding to a cell membrane site, inhibiting its interaction with a matrix site; and b) by binding to a matrix site, inhibiting its interaction with a cell membrane site. If these antibodies are important in interfering with tumor cell invasion and metastasis, this could explain anti-tumor activity in the absence of immune targeting, and is of clear clinical importance.

Description of the in vitro assays

(1) General considerations

Invasion of biological structures by tumor cells takes place over relatively long time frames, and the number of invading cells in a population is very small. These considerations place definite constraints on the assays. Exposure to different matrix components will affect not only invasion, but also proliferative rate and gene expression. In practice, Applicant has adjusted for this proliferation that may take place during an invasive assay but ignored changes in gene expression. Determining the number of invading cells using radioisotopic labeling of the tumor cells yields high experimental variations due to the long duration of the assays (days), differential proliferation rates and the small percentage of the labeled population which will actually invade. Most of these assays are best quantitated by direct visualization and counting of the invading cells. This is tedious but unavoidable.

30 (2) Invasion of transwell barriers impregnated with EHS-derived basement membrane matrix (matrigel).

In this assay a double chamber system is used. Separating the two chambers is a polycarbonate filter with eight micron pores. Applicant used Corning "Transwells" 35 for this assay as these are much simpler to handle than a

Boyden chamber. They consist of 24-well tissue culture plates with removable inserts. The bottom of the insert consists of the polycarbonate filter and the test cells are plated on top of this. 100  $\mu$ l of a 1:20 dilution of commercial Matrigel (Collaborative Research Inc.) is added to the insert on top of the filter. This is allowed to impregnate the filter and to gel, and is then dried down until use. When required, the matrigel is rehydrated for two hours with serum-free medium.  $5 \times 10^4$  test tumor cells are added to the upper well in 200  $\mu$ l of medium. 0.8 ml of medium is added to the lower chamber. The transwells are incubated for 2 to 7 days to allow for the tumor cells to invade through the matrix in the filter and so reach the lower chamber. The exact time required depends on the cell line and must be determined in control experiments. The cultures are fed daily during this time. The upper inserts are then removed and the cells in the lower chamber allowed to grow up to form colonies over the next week. The plates are then washed, fixed in methanol and stained with acid Hematoxylin. The small number of cells invading through the matrix-impregnated filter is essentially clonal, and results from colony counting are identical to those using radiolabeled cell populations. Colony counts are much more reproducible and have lower experimental errors. The number of cells plated in the upper chamber at the start of the experiment does not allow for any extensive proliferation (it is a very small area), and proliferation of cells in the upper chamber is excluded as a meaningful variable. Test antibodies are included in the medium in both chambers until the insert (upper chamber) is removed. At the time that the initial cells are plated, aliquots of cells are also plated in each test medium to look for any unexpected toxicity. These cells are monitored for the time that the upper chambers are in place.

Table 1Effect of Different Ab2s on Tumor Cell Invasion

	Medium	Number of Colonies (invaded cells)	% of Control
5	Control Antibody (MOPC21)	100	
	IM04	ND	
	IM06	18	
	IM08	190	
10	IM09	220	
	IM13	134	
	IM19	344	
	IM20	228	
	IM30	ND	
15	IMelpg2	35	
	MEM136	40	

ND = not done.

Induction of an Ab3 Response in Rabbits

The anti-tumor humoral response induced by different Ab2s in rabbits was studied against a melanoma cell line, Colo38, which expresses MPG. Unrelated isotype-matched control antibody immune rabbit sera was used as a control. FIGURE 9 demonstrates that after two immunizations all the Ab2s were able to induce an anti-anti-idiotype response in rabbits. Applicant then investigated whether any of the sera contained anti-tumor antibodies. Serial dilutions of Ab2 immune sera were incubated for 1 hour with Colo38 cells grown in each well of 96-well plates. The plates were finally developed with goat anti-rabbit IgG. FIGURE 9 shows representative binding data with Ab2 immune sera. As demonstrated, only IM06, IM30 and IMelpg2 induced an anti-tumor response after two immunizations. The binding was specific as the sera did not bind to control MPG-neuroblastoma cell line NMB7. The control sera also did not show appreciable binding to Colo38. Sera collected from rabbits after third and fourth immunizations

contained more anti-tumor antibodies than sera collected after the second immunization.

The data in Table 1 describes the effect of different Ab2s on tumor cell invasion. The results are expressed as a percentage of control colonies invaded. For example, with IM06 only 18% of the control colonies were formed, i.e., there was an 82% inhibition of colony formation. In contrast, 65% inhibition was achieved with IMelpg2. The data demonstrates that both IM06 and IMelpg2 are interfering (via MPG determinant) with the interactions between tumor cells and the basement membrane component that is needed for tumor cell invasion and metastasis. It is possible that IM06 and IMelpg2 bear the MPG image and act as an antagonist for cell and basement membrane interaction. When a direct binding ELISA was performed to determine the presence of anti-MPG antibodies in rabbit sera induced by various Ab2s, Applicant observed that IMelpg2 induced the highest level of Ab3(Ab1') response. See FIGURE 9.

20 Example 3.

In Vitro Evaluation of IMelpg2 and IM06 in Rabbits and Mice Cells

The human melanoma cell lines Colo38, 397, Meljur and 25 M21 (MPG<sup>+</sup>) were grown in RPMI 1640 medium supplemented with 10% calf serum, 1% glutamine and 10 µg/ml gentamicin sulfate. The human neuroblastoma cell line NMB7 and the mammary carcinoma cells MCF7 (MPG<sup>-</sup>) were grown in the same media and used as control cell line. The Colo38 cell line 30 is available from Dr. Soldano Ferrone at New York Medical College. Other cell lines were obtained from ATCC, Rockville, MD.

mAb and conventional antisera

The MoAb1 MEM136 ( $\gamma_1, \kappa$ ) is reactive with a MPG 35 epitope. The MoAb1 225.28 ( $\gamma_2a, \kappa$ ) is reactive with a

different epitope on MPG than that recognized by MEM136, (Wilson *et al.*, *Int. J. Cancer* 28:293 (1981)). The mAb MOPC21 ( $\gamma_1, \kappa$ ; Bionetics, Charleston, SC) was used as an isotype-matched control. The mAb2s, IM04, IM06, IM08, 5 IM09, IM13, IM19, IM20 and IMelpg2, are secreted by hybridomas generated from splenocytes from A/J mice immunized with MEM136. Anti-HLA class I mAb TP25·99 and anti-HLA class II mAb 05/13 were kindly provided by Dr. S. Ferrone of New York Medical College. The MoAb1 MEM136 10 ( $\gamma_1, \kappa$ ) was purified on a protein A-Sepharose 4B column, as were all Ab2s. The purity of these preparations was tested by SDS-PAGE electrophoresis and was >95% as judged by Coomassie blue staining. Immunoreactivity was tested in an ELISA. For RIAs, MoAbs were labeled with  $^{125}\text{I}$  15 utilizing the chloramine T method. FITC-goat anti-mouse IgG antibodies and FITC-goat anti-rabbit IgG antibodies were purchased from Becton-Dickinson, Mountain View, CA.

#### Immunization of mice

Eight wk old BALB/c mice (Jackson Labs., Bar Harbor, 20 ME) (three in each group) were immunized with 50  $\mu\text{g}$  of IM06 or IMelpg2 emulsified with MDP-A. MDP-A is an adjuvant containing a muramyl dipeptide derivative (Allison, *et al.*, *J. Immunol. Methods* 95:157 (1986)). Mice were injected in the footpads, tail-base and multiple subcutaneous ("s.c.") sites. Three subsequent immunizations were given s.c. with the same dose of antigen at 2-wk intervals. Control mice were immunized with 50  $\mu\text{g}$  of MOPC21, an unrelated isotype-matched antibody, with MDP-A. A group of mice were also immunized with  $10^7$  MPG<sup>+</sup> Colo38 25 cells or  $10^7$  MPG<sup>+</sup> NMB7 cells. Mice were bled 7 and 14 days after each immunization.

#### Immunization of rabbits

Adult NZW rabbits were immunized subcutaneously (s.c.) with 500  $\mu\text{g}$  of Ab2s with MDP-A. Rabbits were 35 boosted three times with the same amount of antigen every

2 wks and bled 7 and 14 days after each immunization. Control rabbits were immunized with 500 µg of MOPC21 with MDP-A. A group of rabbits were also immunized with  $10^7$  Colo38 or  $10^7$  NMB7 cells.

5 Serological assays

Indirect immunofluorescence ("IIF") was performed by incubating target cells ( $5 \times 10^5$  cells in PBS supplemented with 0.2% BSA and 0.02% NaN<sub>3</sub>) with dilutions of antisera or purified antibodies for 1 hour at 4°C. After incubation, 10 cells were washed and incubated with FITC-labeled anti-IgG. Thereafter, cells were washed and analyzed on a FACScan (Becton Dickinson).

Radioimmunoassays

The inhibition assay to map determinants recognized by 15 different antibodies was performed by adding <sup>125</sup>I-MEM136, mixed with different amounts of unlabeled antibody without prior incubation, to Colo38 melanoma cells fixed on microtiter plates. After overnight incubation at 4°C, plates were washed and bound radioactivity was counted in 20 a gamma counter. The specificity of the inhibition was analyzed by measuring the ability of test sera to inhibit the binding of <sup>125</sup>I-MEM136 to Colo38 cells. Results are expressed as percent of inhibition of binding of MEM136 to MPG<sup>+</sup> Colo38 cells in presence of control or immune sera as 25 compared to the binding performed in the presence of preimmune sera.

The idiotypic response was analyzed as described earlier (Raychaudhuri, et al., J. Immunol. 139:1743 (1987)). Briefly, 100 ng F(ab')<sub>2</sub> fragments of IM06 or 30 IMelpg2 were bound to microtiter plates for two hours at room temperature. The plates were washed with PBS and then incubated with 1% BSA in PBS for one hour. Serially diluted sera from the different experimental and control groups were incubated in the wells simultaneously in the presence of 10,000 cpm of <sup>125</sup>I-MEM136 for 18 hours at 4°C.

The plates were thoroughly washed and the radioactivity in each well was determined with a gamma counter. The results are expressed as percentages of inhibition of  $^{125}\text{I}$ -MEM136 binding to the  $\text{Ab}_2\text{-F}(\text{ab}')_2$  fragment in presence of 5 control or immune sera, as compared to the binding performed in the presence of preimmune sera.

#### Affinity determination

Dissociation constants were determined by performing the Scatchard analysis of equilibrium binding of  $^{125}\text{I}$ -MEM136 10 to different Ab2s according to the procedure of Wilson and Lentz, *Biochemistry*, 27:6667 (1988). Briefly, wells of 96-well plates coated with Ab2s (100 ng/well) were incubated with different concentrations of  $^{125}\text{I}$ -MEM136 for 18 hours at 4°C. After incubation, an aliquot of the  $^{125}\text{I}$ - 15 MEM136 solution was removed and placed in a gamma counter to determine the amount of free  $^{125}\text{I}$ -MEM136. The plates were then washed and dried and the amount of bound  $^{125}\text{I}$ -MEM136 per well was determined.

#### Affinity purification of Ab3 antibodies.

20 Immune rabbit sera, diluted (1:5) in borate buffered saline ("BBS", pH 8.0), were first passed through an adsorbent containing bound normal mouse IgG to eliminate the anti-isotypic antibodies. Sera were subsequently applied to an Ab2-Sepharose 4B affinity column. The bound 25 antibodies were eluted with 0.1 M acetic acid containing 150 mM sodium chloride and the eluates neutralized immediately. These anti-anti-idiotypic antibodies (Ab3s) were examined for their ability to bind to Colo38 cells. As control, sera from rabbits immunized with MOPC21 were 30 purified on MOPC21-Sepharose 4B column.

#### Ab3 specificity assays

Purified Ab3s were incubated with Colo38 ( $\text{MPG}^+$ ) and NMB7 ( $\text{MPG}^-$ ) cell lines as described (Jensen, P.E., *J. Exp. Med.*, 171:1779 (1990)). Briefly, Colo38 and NMB7 cells

were fixed with 1% paraformaldehyde for 30 min. at room temperature and washed thoroughly. The purified Ab3 (100 µg) was incubated with these fixed Colo38 and NMB7 cells (100 x 10<sup>6</sup>) for 2 hours at room temperature. The degree of absorption was determined by IIF analysis on Colo38 cells using Ab3s before and after absorption with Colo38 and NMB7 cells.

To determine the cross-reactivity of IMelpg2-Ab3 (Ab3 purified from IMelpg2 immune rabbit sera) with IM06-Ab3 (Ab3 purified from IM06 immune rabbit sera), IMelpg2-Ab3 was absorbed on IM06-Sepharose 4B absorbent. After absorption, both bound and unbound fractions were tested for anti-melanoma and anti-idiotypic activity. For the determination of Ab1' reactivity, various fractions were analyzed in IIF and also inhibition of <sup>125</sup>I-MEM136 binding to Colo38 was determined in the presence of various fractions. Anti-idiotypic reactivity was determined as described earlier. Different concentrations of Ab3 purified from different rabbits (IM06 and IMelpg2 immune rabbit sera) were incubated with <sup>125</sup>I-IMelpg2-Ab3 (60,000 cpm/well) in IMelpg2-F(ab')<sub>2</sub> coated (100 ng/well) plates for 18 hours at 4°C. The same technique was employed to analyze different dilutions of mouse sera. The plates were washed, dried and counted in a gamma counter. For the determination of the Ab1' cross-reactivity, the binding of <sup>125</sup>I-IMelpg2-Ab3 to Colo38 was measured in the presence of the same fractions described above.

Induction of Ab3 response in rabbits

The goal of this study was to identify a mouse Ab2 which would induce an anti-MPG response across the species barrier and react with MPG<sup>+</sup> melanoma cells. Applicant examined the induction of Ab3 and Ab1' responses in rabbits by different monoclonal Ab2s generated against MEM136. Table 2 describes the name, isotype and relative affinity of different Ab2s. The anti-tumor humoral response induced by different Ab2s with MDP-A in rabbits

was assessed using the MPG-positive melanoma cell line, Colo38. As a control, rabbits received an isotype-matched control antibody, MOPC21. After two immunizations, all Ab2s induced Ab3 responses in rabbits. However, only sera 5 from rabbits immunized with IM06 and IMelpg2 produced significantly higher binding to melanoma cells compared to the binding observed with isotype matched MOPC21 immune sera. The binding was specific as the sera did not bind to a control neuroblastoma cell line, NMB7.

10 Table 2Characteristics of Ab2s used in this Study

Name of Ab2	Isotype	Relative Affinity to MEM 136 <sup>a</sup>	IC <sub>50</sub> <sup>b</sup> of MEM136 binding to melanoma cells
IM04	γ <sub>1,κ</sub>	1.01 x 10 <sup>-10</sup>	1 μg/ml
IM08	γ <sub>1,κ</sub>	2.20 x 10 <sup>-10</sup>	<1 μg/ml
IMelpg2	γ <sub>1,κ</sub>	5.20 x 10 <sup>-10</sup>	1 μg/ml
IM06	γ <sub>1,κ</sub>	2.30 x 10 <sup>-10</sup>	2 μg/ml
IM09	γ <sub>1,κ</sub>	1.40 x 10 <sup>-10</sup>	16 μg/ml
IM13	γ <sub>1,κ</sub>	1.30 x 10 <sup>-10</sup>	2 μg/ml
IM19	γ <sub>1,κ</sub>	2.20 x 10 <sup>-10</sup>	>16 μg/ml
IM20	γ <sub>2a,κ</sub>	0.74 x 10 <sup>-10</sup>	2 μg/ml

<sup>a</sup> 15 Affinity of Ab2s to MEM136 was determined as follows:  
25 Different concentrations of <sup>125</sup>I-MEM136 were added to each well preincubated with Ab2 (100 ng/well) for 18 hours at 4°C. A count of 10 μl aliquot from each well was taken to determine the free <sup>125</sup>I-MEM136. The plates were washed and the bound <sup>125</sup>I-MEM136/well determined. Affinity was measured by the Schatchard analysis of the equilibrium binding of <sup>125</sup>I-MEM136 to different Ab2s, and Kd values in molar quantity were determined.

<sup>b</sup> 30 IC<sub>50</sub> is the concentration of an Ab2 required for a fifty percent inhibition of <sup>125</sup>I-MEM136 binding to the melanoma cell line Colo38.

ELISA binding data were confirmed by IIF analysis using IMelpg2 and IM06 immune rabbit sera obtained seven days after the fourth immunization (FIGURE 10). Colo38 and NMB7 cells were incubated with different dilutions of sera washed and subsequently labelled with fluorescein-conjugated goat anti-rabbit IgG. Panel (a) IMelpg2 immune sera on Colo38, (b) IMelpg2 immune sera on NMB7, (c) IM06 immune sera on Colo38, and (d) IM06 immune sera on NMB7. Sera of rabbits immunized with MOPC21 in MDP-A were employed as control, panel (e). The data presented in the figure were obtained with serum dilutions of 1:500. Sera from rabbits immunized with either IM06 or IMelpg2 bound only to MPG<sup>+</sup> Colo38 cells and not to MPG<sup>-</sup> NMB7 cells. Use of the MDP-A adjuvant was required for the effective induction of Ab3 responses, in agreement with previous observations (Chattopadhyay, et al., *J. Immunol.*, submitted (1990)). To demonstrate that the anti-anti-id immune sera only react to MPG<sup>+</sup> cells, the IMelpg2 immune sera was further tested against various MPG<sup>+</sup> and MPG<sup>-</sup> cells. Different MPG<sup>+</sup> cells, Colo38, M21, 397, Meljur and MPG<sup>-</sup> cells, NMB7 and MCF-7 were incubated with 1:500 dilution of serum washed and labeled with fluorescein-conjugated goat anti-rabbit IgG. Results shown in FIGURE 11 demonstrate that the reactivity of IMelpg2 immune sera co-type with MPG expressing cell lines.

Total Ab3 response was analyzed by a homologous inhibition assay where binding of <sup>125</sup>I-MEM136 to F(ab')<sub>2</sub> fragments of IMelpg2 or IM06 was studied at different dilutions of IMelpg2 or IM06 immune sera. Sera from rabbits immunized with MOPC21 in MDP-A were used as control. The results are expressed as percentages of inhibition which are arithmetic means of triplicate determinations. As shown in FIGURE 12, both IMelpg2 and IM06 induced Ab3 responses in rabbits of similar magnitude. A 50% inhibition of <sup>125</sup>I-MEM136 binding to IMelpg2 was achieved at a 1/250 dilution of sera from rabbits immunized with IMelpg2. On the other hand, a 1/150

dilution of sera from rabbits immunized with IM06 was required to produce a similar level of inhibition. Next, Applicant demonstrated that both IMelpg2 and IM06 immune sera inhibited the binding of  $^{125}\text{I}$ -MEM136 to Colo38 (FIGURE 5 13). The Ab1' response was analyzed by inhibition of binding of  $^{125}\text{I}$ -MEM136 to Colo38 cells using different dilutions of sera. The anti-mouse activity was eliminated by passing the rabbit sera through normal mouse IgG-sepharose 4B. The results are expressed as percentages of 10 inhibition which are arithmetic means of triplicate determinations. Sera from rabbits immunized with MOPC21 in MDP-A were used as control. A 50% inhibition of binding of MEM136 to Colo38 was achieved at approximately a serum dilution of 1:100. These results demonstrate that 15 monoclonal anti-id can be used to induce tumor specific anti-tumor responses in rabbits that are idiotypically cross-reactive with the Ab1 and also that the Ab1 and Ab1' are recognizing the same epitope on the MPG antigen. Therefore, no significant difference in total Ab3 and Ab1' 20 responses could be seen in rabbit sera immunized with IMelpg2 and IM06.

Induction of Ab3 Response in Mice.

Having established that both IMelpg2 and IM06 are capable of inducing anti-MPG response, in an xenogeneic 25 situation, Applicant investigated whether these Ab2s induced similar responses in an allogeneic system. BALB/c mice were immunized with the A/J-derived IMelpg2 and IM06 in MDP-A. Mice were bled and tested for Ab3 and Ab1' response. The results are shown in FIGURES 14, 15 and 16. 30 The data in FIGURE 14 demonstrate that IMelpg2 and IM06 induced Ab3 responses in BALB/c mice. The results are expressed as percentages of inhibition which are arithmetic means of triplicate determinations. Sera of mice immunized with MOPC21 in MDP-A were employed as control. 35 A 1:300 dilution of sera from mice immunized with IMelpg2 in MDP-A inhibited 50% of binding of mAb MEM136 to IMelpg2

and a 1:90 dilution of sera from mice immunized with IM06 was required to produce the same inhibition of binding. However, as shown in FIGURE 15, IIF analysis demonstrated that, in mice, only IMelpg2 induced an antibody response reactive with the melanoma cell line, Colo38. Colo38 and NMB7 cells were incubated with different dilutions of sera and labeled with fluorescein-conjugated goat anti-mouse IgG. Panel (a) shows the binding of IMelpg2 immune sera on Colo38, (b) represents binding of IMelpg2 immune sera on NMB7; panels (c) and (d) demonstrate the binding of IM06 immune sera to Colo38 cells and NMB7 cells, respectively. Sera of mice immunized with MOPC21 in MDP-A were employed as control (e). The data presented in the figure were obtained with a serum dilution of 1:50. Reliable binding of IMelpg2 Ab1' antisera to Colo38 cells could be detected in 6/6 mice tested, even at a serum dilution of 1:1000. In contrast, mice immunized with IM06 in MDP-A did not develop antibodies reacting with melanoma cells even at a dilution of 1:50. The binding of the Ab1', induced by IMelpg2, was specific, as demonstrated by the inability of immune sera to bind to MPG<sup>+</sup> NMB7 cells and also by the inability of control MOPC21 immune sera to bind to Colo38 cells.

To compare the epitope specificity of Ab1 and the induced Ab1', Applicant tested whether IMelpg2 immune sera can inhibit the binding of <sup>125</sup>I-MEM136 to Colo38 cells. The results are shown in FIGURE 16. The Ab1' response was analyzed by inhibition of binding of <sup>125</sup>I-MEM136 to Colo38 cells by different dilutions of sera. The results are expressed as percentages of inhibition which are arithmetic means of triplicate determinations. Sera from mice immunized with MOPC21 were used as control. A 50% inhibition of binding of MEM136 to Colo38 was achieved at a 1/400 dilution of sera. Applicant's results indicate that a monoclonal anti-id can be used to induce anti-tumor responses in allogenic mice which are idiotypically cross-reactive with the Ab1. Our results also suggest that Ab1

and Ab1' are recognizing the same epitope on the MPG antigen.

Affinity Purified Rabbit Ab3 Contains Anti-MPG Reactivity

To demonstrate that the anti-anti-idiotype antibodies 5 (Ab3s) are the active component in the IMelpg2 and IM06 immune sera, Ab3s were purified from immune sera. Colo38 and NMB7 cells were incubated with 10 µg of Ab3 purified from rabbits immunized with IMelpg2 or IM06 in MDP-A. The results are shown in FIGURE 17, Panels (a) and (b) show 10 profiles obtained by incubating IMelpg2-Ab3 with Colo38 or NMB7 cells while panels (c) and (d) shows the profiles obtained with IM06-Ab3 incubated with Colo38 or NMB7 cells. Panel (e) shows the profile obtained with 10 µg of MOPC21. The data presented in FIGURE 17 suggest that 15 purified Ab3s from IMelpg2 and IM06 immune sera bind to Colo38 (MPG<sup>+</sup>) and not to NMB7 (MPG<sup>-</sup>) cells. As expected, these purified Ab3s were found to be idiotypically cross-reactive with the Ab1 as determined by the ability of these Ab3s to inhibit binding of <sup>125</sup>I-MEM136 to IMelpg2 or 20 IM06. The specificity of these Ab3s was further supported by absorption of Ab3s on Colo38 and NMB7 cells. FIGURE 18 shows that the Ab1' response is depleted when IMelpg2-Ab3 was absorbed with Colo38 cells but not with NMB7 cells. Similar results were observed with IM06-Ab3. Finally, as 25 shown in FIGURE 19, the cross-reactivity of epitope specificity of IMelpg2-Ab3, IM06-Ab3 and the Ab1 was demonstrated by the ability of purified Ab3 to inhibit the binding of MEM136 to Colo38. The percentages of inhibition were computed and the average obtained with two 30 rabbits is shown. MOPC21 anti-i antibodies were used as control.

Fine specificity of mice and rabbits Ab3s

For the qualitative analysis of the Ab3s induced by IMelpg2 and IM06 in mice and rabbits, Applicant analyzed 35 <sup>125</sup>I-IMelpg2-Ab3 binding to IMelpg2 in the presence of

different concentrations of purified IMelpg2-Ab3, IM06-Ab3 and MOPC21 anti-id antibody. Both the murine IMelpg2<sup>-</sup> and IM06<sup>-</sup> immune sera were also used in the same inhibition analysis. A typical inhibition curve is shown in FIGURE 5 20 demonstrating that IMelpg2 induces idiotypically similar Ab3s in mice and rabbits. In (a), MOPC21-anti-id was used as a control; in (b), MOPC21-immune mouse sera was used as a control. Furthermore, in rabbits, IMelpg2 induces Ab3s which are very similar to those induced by 10 IM06. However in mice, IM06-induced Ab3s did not compete well with <sup>125</sup>I-IMelpg2-Ab3 binding to IMelpg2, confirming the notion that, in mice, IMelpg2 and IM06 are behaving immunologically differently.

To further dissect idiotypic crossreactivity between 15 IMelpg2-Ab3 and IM06-Ab3 population in rabbits, IMelpg2-Ab3s were absorbed on IM06-Sepharose column. It should be stated that 10-20% of the IMelpg2-Ab3 population was crossreactive with IM06 idiotype because that was the amount bound to IM06 column. The fractions bound and 20 unbound on IM06 idiotypes were examined for tumor cells binding and for their ability to inhibit the binding of MEM136 to IMelpg2 or melanoma cell line, Colo38. As shown in FIGURE 21, IIF analysis was performed using IMelpg2-Ab3, before and after absorption on IM06-sepharose against 25 (a) Colo38 (MPG<sup>+</sup>) cells and (b) NMB7 (MPG<sup>-</sup>) cells. The fluorescein-conjugated goat anti-rabbit IgG was used for staining. These results demonstrate that the majority of Ab1<sup>+</sup> fraction in the IMelpg2-Ab3 population remains in IM06 unbound fraction. That is, the majority of the Ab1<sup>+</sup> 30 fraction in IMelpg2-Ab3 population is idiotypically non-crossreactive with IM06. FIGURES 22 and 23 confirm the tumor cell binding study that indeed the whole IMelpg2-Ab3 population and the population depleted of IM06 idiotype contain crossreactive populations with the Ab1, MEM136. 35 However, from FIGURES 22 and 23, it is also apparent that IMelpg2-Ab3 population bearing IM06 idiotype is to a lesser extent cross-reactive with MEM136.

Discussion

Applicant analyzed the ability of eight Ab2s to induce anti-MPG responses in mice and rabbits cross-reacting with human melanoma cells. All eight Ab2s are 5 idiotypically crossreactive and are specific for their common Ab1, MEM136 combining site<sup>3</sup>. Applicant initially immunized rabbits with all eight Ab2s for the induction of anti-MPG responses. Out of eight Ab2s tested, only IMelpg2 and IM06 induced anti-MPG responses in rabbits.

10 On the other hand, both IMelpg2 and IM06 induced equivalent amount of Ab3 responses in allogeneic mice. However, in allogeneic mice only IMelpg2 induced anti-MPG responses.

Earlier studies characterized various types of Ab2s 15 obtained after immunization with an Ab1 (Jerne, et al., EMBO J., 1:243 (1982); Bona, et al., Monoclonal and Anti-idiotypic Antibodies: Probes for Receptor Structure and Function, p. 141 (1984)). The internal image or Ab2 $\beta$  class of antibodies is believed to mimic serologically an 20 epitope on the antigen used to generate the original Ab1 and can be used to generate an antigen specific response in place of nominal antigen. This class of Ab2s is also capable of inducing an antigen specific response across the species barrier. Alternatively, the non-internal 25 image class of Ab2s (Ab2 $\alpha$ ) may or may not be directed against a combining site related idiootope and will not induce an antigen specific response across the species barrier. Based upon their serologic characteristics, IMelpg2 may belong to the internal image class of 30 antibodies while IM06 may belong to the non-internal image class of antibodies. The Ab3 response induced by IMelpg2 is idiotypically crossreactive with it's Ab1 (Ab3s inhibited the binding of MEM136 to IMelpg2). Further, the anti-MPG response induced by IMelpg2 in mice and rabbits 35 reacted to MPG $^+$  melanoma cells and also inhibited the binding of Ab1 to Colo38. Therefore, the Ab1 and the Ab1'

response induced by IMelpg2 share similar antigenic specificity.

The Ab3 response induced by IM06 in mice and rabbits is idiotypically crossreactive with MEM136, as demonstrated by the ability of Ab3s, from both mice and rabbits, to compete with MEM136 to bind to IMelpg2 or IM06. However, IM06-immune mouse sera neither bound to Colo38 cells nor competed with MEM136 to bind to Colo38 cells. To further characterize the serologic mimicry between the MPG epitope and the Ab2s, anti-Colo38 sera from mice and rabbits were tested in a direct binding RIA to determine whether they bound to IM06 or IMelpg2 and also were tested to determine whether they could compete with MEM136 to bind to Colo38, IMelpg2 or IM06. No detectable binding to Ab2s or inhibition of MEM136 to Ab2s by the sera could be observed. Therefore, although Colo38 cells contain a high density of MPG molecules on their surface, there is no evidence for an anti-MPG response in Colo38 immunized rabbit sera which is idiotypically cross-reactive with MEM136. Although immune sera from animals immunized with purified MPG antigen were not examined, Ab2s are apparently activating silent clones as has been demonstrated by others (Bona, et al., J. Exp. Med., 153:951 (1981); Hiernaux, et al., J. Exp. Med., 153:1004 (1981)). The inability of IM06 to induce an anti-MPG response in mice could be due to the fact that mice lack the IM06 idioype. Alternatively, IM06 may be inducing a regulatory mechanism which interferes with its ability to induce id<sup>+</sup>Ag<sup>+</sup> responses.

Recently, Shearer et. al., J. Immunol., 145:932 (1990) generated four monoclonal Ab2s against a monoclonal anti-SV-40-T antigen that are idiotypically crossreactive. They examined only one Ab2 for the induction of anti-SV-40 antibody response. They argued that as all Ab2s are crossreactive, one Ab2 should represent all. Applicant's data indicate that Ab2s against an Ab1 can be highly crossreactive, yet differ drastically in their ability to

induce antigen specific responses in different species. Therefore, to identify a potential idiotype-based active immunotherapeutic agent, one ought to immunize various Ab2s in various species as described earlier (Nisonoff, et al., Clin. Immunol. Immunopathol., 21:397 (1981)).

The induction of Ab3 responses in rabbits by IMelpg2 and IM06 warrants some discussion. The Ab3s induced by these two Ab2s are idiotypically crossreactive (i.e. the Ab3s inhibited the binding of IMelpg2-Ab3 to IMelpg2). 10 Applicant's data also indicate that both Ab3s are recognizing an epitope defined by the Ab1 (i.e. the Ab3s inhibited the binding of MEM136 to melanoma cells). However when the IMelpg2-induced Ab3 was separated on an IM06 column, two fractions were obtained. Eighty to 15 ninety percent of the Ab3 population did not bind to IM06 column (IM06 id<sup>-</sup>) and contained the majority of the IMelpg2-Ab3 idiotype and Ab1' reactivity. Ten to twenty percent of the IMelpg2-Ab3 population bound to IM06 (IM06 id<sup>+</sup>) inhibited poorly the binding of MEM136 to IMelpg2 or 20 melanoma cells (i.e., contain little to no Ab1'). Similar results were observed with IM06 immune rabbit sera indicating that the majority of the IM06-Ab3s in rabbits are IM06 id specific and contained Ab1' reactivity. Therefore, two Ab2s made against the same Ab1 induced a 25 different population of Ab3 response. The IMelpg2-specific idiotype (IMelpg2 id<sup>+</sup> IM06 id<sup>-</sup>) may contain an antigen image or regulatory components necessary for the induction of anti-TAA response across the species barrier. The weak inhibition of MEM136 binding to IMelpg2 or Colo38 30 cells by the (IM06 id<sup>+</sup> IMelpg2 id<sup>+</sup>) population could be due to the low affinity of crossreactive Ab3 population for IMelpg2 and for the MPG antigen on Colo38 cells.

In summary, Applicant analyzed the Ab3 response in rabbits by eight highly crossreactive Ab2s generated 35 against an MoAb1. Only two, IM06 and IMelpg2, induced specific anti-MPG responses. When tested in mice, only IMelpg2 induced specific anti-MPG responses. The majority

of the Ab3 and Ab1' reactivity in the IMelpg2-Ab3 population in rabbits could be attributed to the IMelpg2 id<sup>+</sup> IM06 id<sup>-</sup> population. The epitope specificity of Ab1' induced by IMelpg2 in mice and rabbits is similar to the original 5 Ab1, MEM136. The Ab2, IMelpg2, is a potentially important active immunotherapeutic agent in melanoma patients. Collectively, Applicant's data indicates that IMelpg2 (hybridoma cell line producing the IMelpg2 antibody deposited with the ATCC on October 18, 1989, ATCC designation 10 No. HB 10265) is the most promising candidate for inducing an anti-tumor response in active immunotherapy.

Example 4

Immunological Equivalents of IMelpg2

This invention is not limited to IMelpg2. Indeed, 15 many anti-idiotypic antibodies can be made that are directed to the idotype of the murine monoclonal antibody MEM136. Any monoclonal antibody secreted by hybridomas from any species, as well as any polyclonal (*i.e.*, conventional antisera from any species), with the same immunological specificity or specificity may be used. It also 20 will be appreciated by those skilled in the art that derivatives of the murine monoclonal MEM136 may also be used. As used herein, the term "derivative" includes (but is not limited to) related cell lines as well as any antibody 25 or antibody fragments including synthetic peptides that may compete, completely or partially with monoclonal antibody MEM136 for binding to MPG.

To practice this invention with anti-idiotype antibodies equivalent to IMelpg2, the first step is to 30 identify idiotypic components reactive with the specific determinant (epitope) of a MPG antigen. This may be accomplished by any of several approaches known to those skilled in the art. For example, similar antibodies (Ab1s) with specificity for the MPG antigen epitope recognized 35 by MEM136 may be obtained from melanoma patients, experimental animals immunized with human melanoma cells,

- from commercial sources, or by methods routinely practiced by those skilled in the art. For purposes of this invention, the Ab1 may be either polyclonal or monoclonal antibodies. Polyclonal antibodies can be isolated from patients' or animals' sera by affinity purification using immobilized disease associated antigens as described by Real, *et al.*, *J. Exp. Med.*, 160:1219 (1984) and in the *Handbook of Experimental Immunology* (ed. Weir, *et al.*, (1986)) (hereinafter referred to as "the Handbook").
- 5 Monoclonal antibodies can be generated by standard somatic cell hybridization rescue fusion ("rescue fusion") using standard techniques practiced in the prior art (see the Handbook, *supra* and Carroll, *et al.*, *J. Immunol. Meth.*, 89:61 (1986)) and utilizing patients' peripheral B cells,
- 10 or any other B-cells sensitized to a MPG antigen. Polyclonal and monoclonal antibodies can also be generated using the techniques described above from human subjects or experimental animals inoculated with tumor, with tumor extracts, purified MPG or peptides derived from MPG.
- 15
- 15 These MEM136 equivalent Ab1 antibodies (*i.e.*, those that compete with IMelpg2 for binding to the MPG) could be utilized to generate and identify anti-id Ab2s with immunological specificity identical or similar to IMelpg2.

Example 5

25 Use of the Anti-idiotype Antibodies in Diagnosis, Monitoring and Immunotherapy

The anti-idiotypic antibodies of this invention may be used in disease diagnosis and monitoring, and in active or passive immunotherapy according to the following 30 methods. (Although the following discussion will describe the use of IMelpg2 in disease diagnosis and monitoring and in active or passive immunotherapy, those skilled in the art will recognize that the methods apply, as well, to IMelpg2 equivalents).

35 In disease diagnosis and monitoring IMelpg2 may be incorporated into in vitro assays (such as ELISA or RIA)

in order to measure the levels of serum idiotypes reactive with IMelpg2 before, during and after immunotherapy; for example, in measuring the booster effect of multiple administrations of IMelpg2 in active immunotherapy.

5        In active immunotherapy, IMelpg2 may be administered in order to heighten the immune response to stimulate beneficial components of the immune system; for example, in treatment of malignant melanoma, it is desirable to elicit humoral and cellular responses directed against the  
10 melanoma associated antigen. More specifically with respect to melanoma, it is known that tumor cells traverse extra cellular matrix ("ECM") during the process of invasion and metastasis. The ECM that is most relevant to metastasis is the basal lamina or basement membrane.  
15 Basement membranes are composed mainly of collagen type IV meshed laminin, entactin, fibronectin and proteoglycans. A majority of experimental evidence suggests a close correlation between extracellular membrane degradation and tumor cell invasion and metastasis. Therefore, Ab3  
20 attachment to ECM may interfere with the biology of tumor growth and metastasis by inhibiting the binding of tumor cells to ECM. Taken together, Applicant's data demonstrate the utility of IMelpg2 as an active melanoma immunotherapeutic agent. IMelpg2 also may be useful  
25 inducing protective immunity.

When used in active immunotherapy, the anti-idiotype antibodies of the present invention are preferably administered in an adjuvant formulation meaning adjuvants, carriers, vehicles and conjugation techniques known or developed by those skilled in the art. Such formulations may incorporate Syntex Adjuvant Formulation-1 (SAF-1), muramyl dipeptide derivatives, lipopolysaccharides, pluronic polymers, Bacillus Calmette-Guérin ("BCG"), liposomes, mineral oil emulsions, alum adjuvants, such as aluminum hydroxide and aluminum phosphate, saponins such as Quill A, immune-stimulating complexes ("ISCOMS"), lipid A, keyhole limpet hemocyanin (KLH), hepatitis core anti-

gen, tetanus toxoid, water-in-oil emulsions, glutaraldehyde crosslinking and other conjugation procedures to conjugate the anti-idiotype antibodies to adjuvants or carriers. Examples of adjuvant formulations incorporating 5 these materials and processes have been described in Steinberger, et al., Experimental Parasitology, 58:223-229 (1984); Morein, Nature 332: 287-288 (1988); Allison, J. Immunol. Methods 95:157-168 (1986); Clarke, et al., Nature 330:381-384 (1987); Raychaudhuri, et al., J. Immunol. 137:1743 (1986); Eskola, et al., New Eng. J. Med. 317:717-722 (1987); Allison, Bio/Technology 5:1041 (1987); Leonard, et al., Blood 65:1149-1157 (1985); Practical Guide for Use in Affinity Chromatography and Related Techniques (2nd ed. 1983 by Reactiff IBF) pub. Societe 10 Chemique Pointet-Girard, France.

When used in active immunotherapy, the antibodies are administered to the patient in a manner and dose that will induce tumor regression. They generally will be administered by subcutaneous or intramuscular (IM) injection. 20 The proper dosage of Ab2 to be administered to a particular patient will depend on the patient's size and the status of their immune system function. Dosages may range from 500 µg to 5 mg. The preferred dosage range to be administered would be 100 µg to 5 mg per injection site. 25 Of course, the objective is to use the minimum dose shown to provide efficacious humoral and/or cellular anti-tumor responses. Multiple injection sites may be used.

With regard to the use of the antibodies of this invention in passive immunotherapy, there may be instances 30 of disease (for example, auto-immune disease) in which MPG is a target of deleterious B-cell or T-cell immune responses that are attacking healthy tissue. Passive immunotherapy may be used in this circumstance of disease. When used in passive immunotherapy, the antibodies herein 35 disclosed are not used in adjuvant formulations. The method of administration is intravenous injection and generally, larger doses of antibody are given (in the

range of 50 mg to 5 g). Other uses of these antibodies in passive immunotherapy will be apparent to those skilled in the art. Generally, passive immunotherapeutic uses of these antibodies will be appropriate whenever the MPG is 5 the target of immune responses that one wishes to suppress.

Claims

1. An anti-idiotypic antibody, antibody fragment, peptide, or antisera capable of reacting with at least one of the idiotypes of:

5 (a) murine monoclonal antibody MEM136 and derivatives thereof;

(b) a monoclonal antibody having the same immunological specificity as antibody MEM136; or

(c) polyclonal antibodies having the same

10 immunological specificity as antibody MEM136,

wherein (a), (b), or (c) is capable of reacting with a specific determinant of a MPG antigen.

2. An anti-idiotypic antibody capable of reacting with at least one of the idiotypes of the murine mono-  
15 clonal antibody MEM136, wherein said MEM136 is capable of reacting with a specific determinant of a MPG antigen.

3. An anti-idiotypic antibody capable of reacting with an idioype of murine monoclonal antibody MEM136, or to immunological equivalents of said antibody MEM136.

20 4. An anti-idiotypic antibody raised in a monoclonal or polyclonal system to at least one of the idiotypes of: anti-MPG antigen antibody MEM136; or antibodies capable of competing with said antibody MEM136 for reaction with said MPG antigen.

25 5. An anti-idiotypic antibody capable of: reacting with anti-MPG antigen antibody MEM136, or to antibodies capable of competing with said antibody MEM136 for reacting with said MPG antigen; and inducing the formation of antibodies to a tumor antigen in the same or different  
30 species.

6. The murine anti-idiotypic antibody IMelpg2 and antibodies having the same immunological specificity as said antibody IMelpg2.
7. An anti-idiotypic antibody capable of:
  - 5 (a) reacting with antibodies made against a peptide derived from MPG antigen or antibodies made against IMelpg2; and
  - (b) inducing the formation of antibodies to a tumor antigen.
- 10 8. A anti-idiotype antibody producing hybridoma cell line deposited with the ATCC, Rockville, MD, under accession No. HB 10265.
9. A monoclonal antibody which reacts with and competes for binding to the same anti-MPG antigen as a 15 monoclonal antibody produced by the hybridoma cell line of Claim 8.
10. A monoclonal antibody produced by the cell line of Claim 8.
11. A method of using said anti-idiotype antibody of 20 claim 1 in diagnosis, monitoring, or therapy of cancer.
12. The method of claim 11, wherein said cancer is malignant melanoma.

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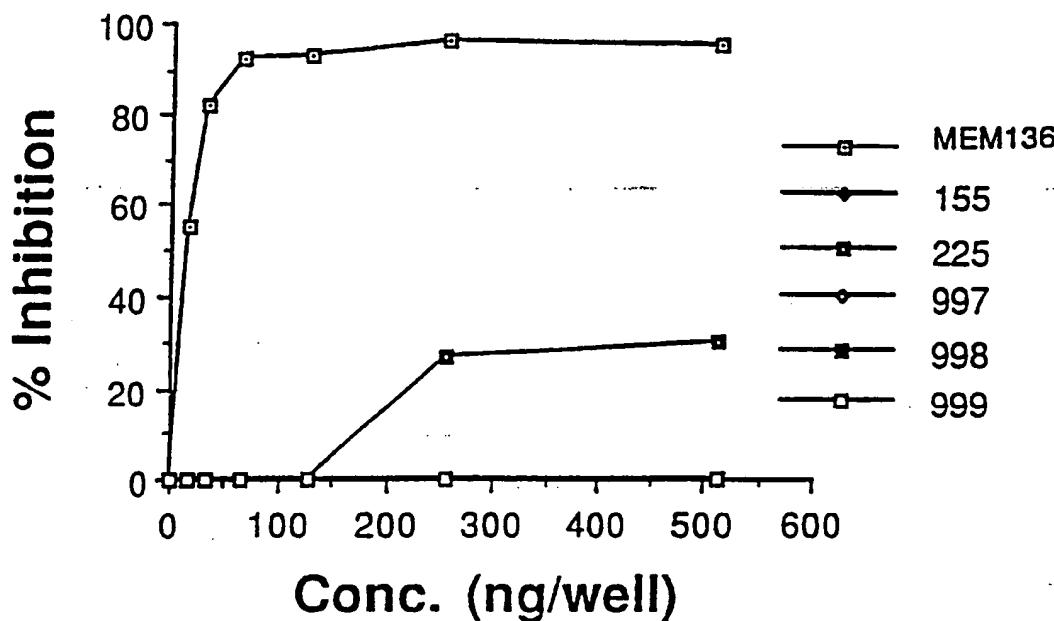
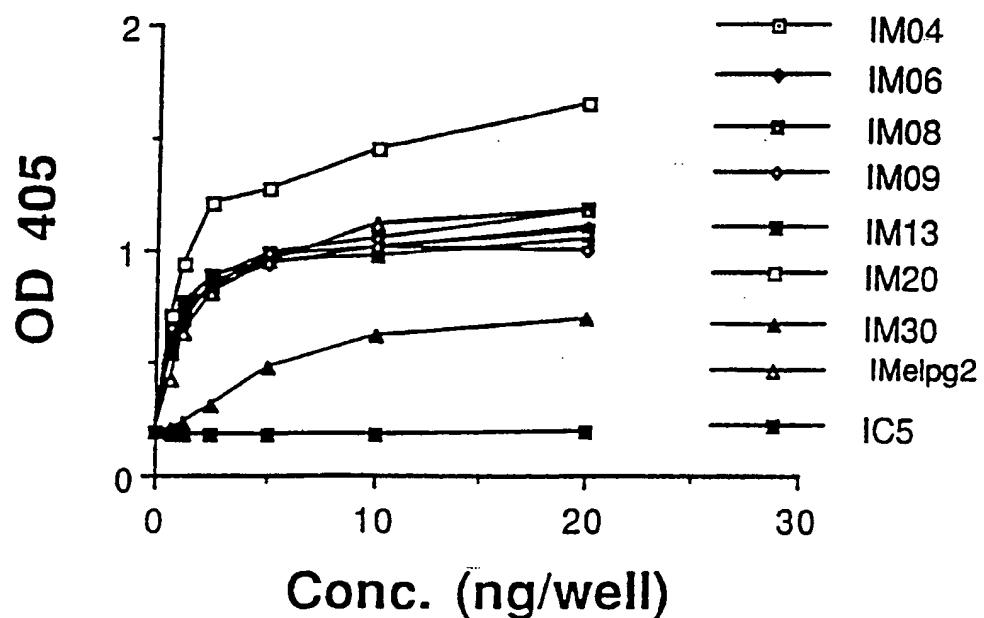


FIG. 1.

FIG. 2.  
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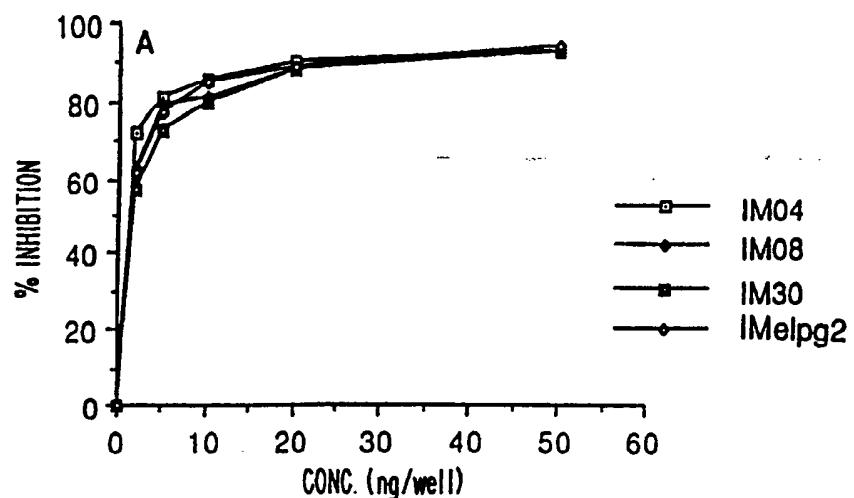


FIG. 3a.

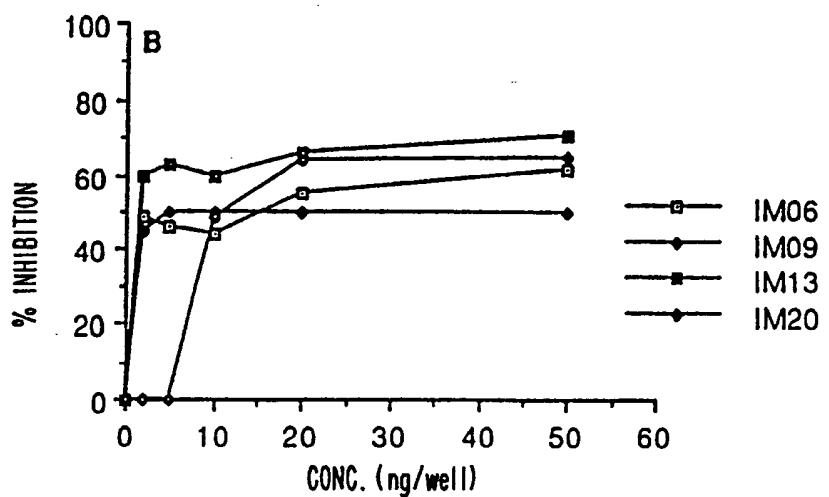


FIG. 3b.

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—○— MEM136  
—●— 155  
—■— 225  
—◆— 997  
—■— 998  
—□— 999

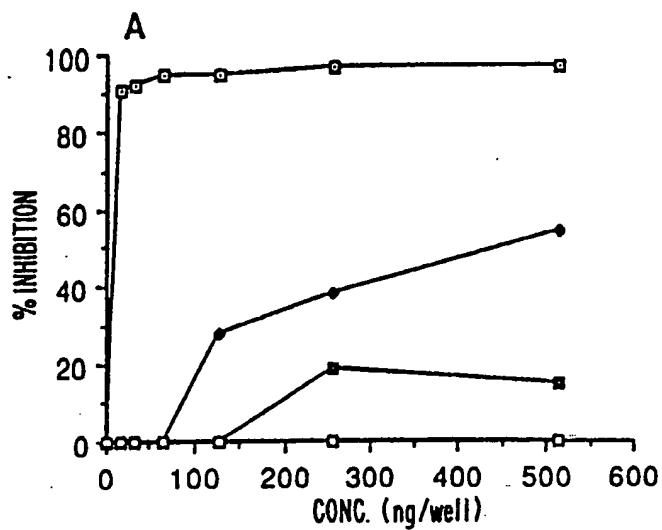


FIG. 4a.

—○— MEM136  
—●— 155  
—■— 225  
—◆— 997  
—■— 998  
—□— 999

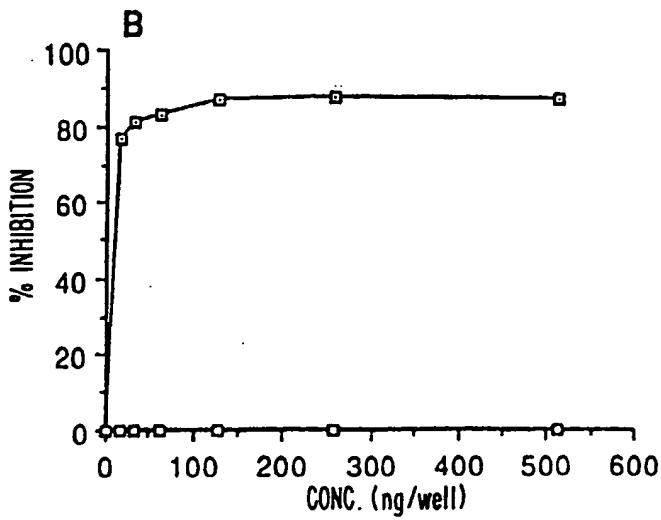


FIG. 4b.

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FIG. 5a.

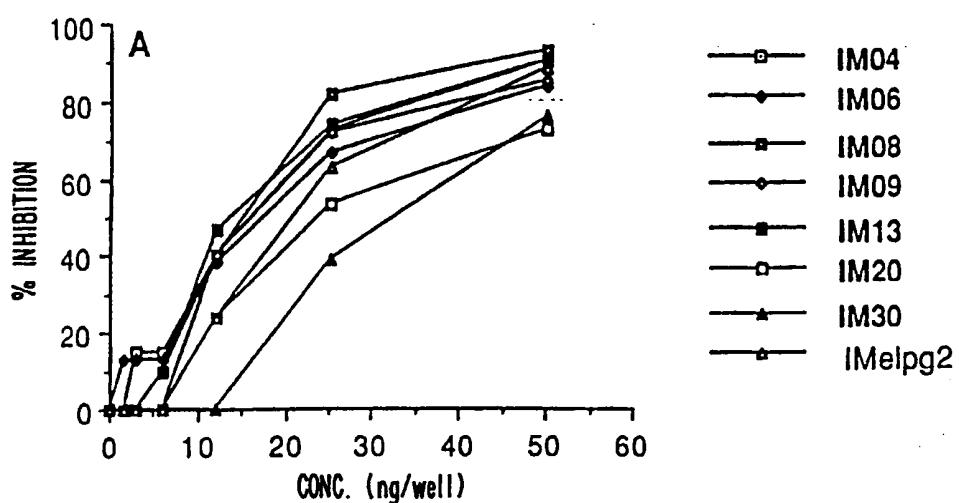
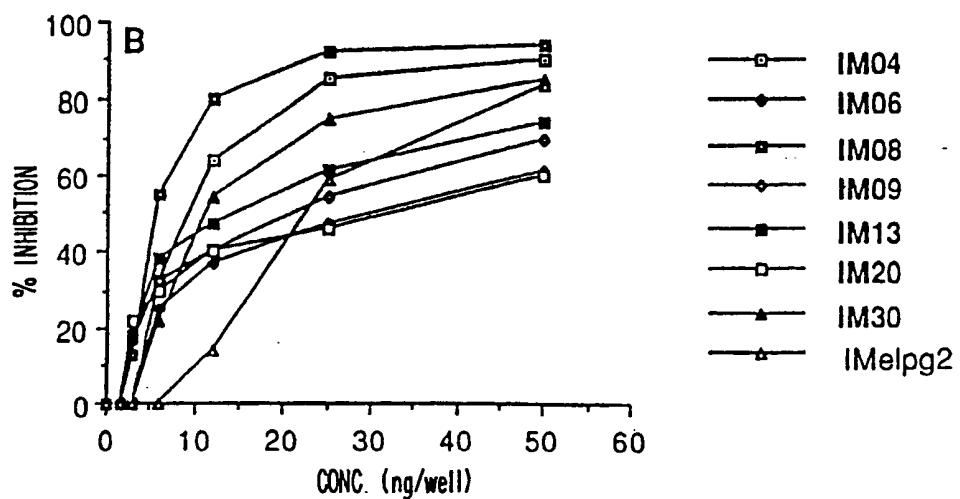
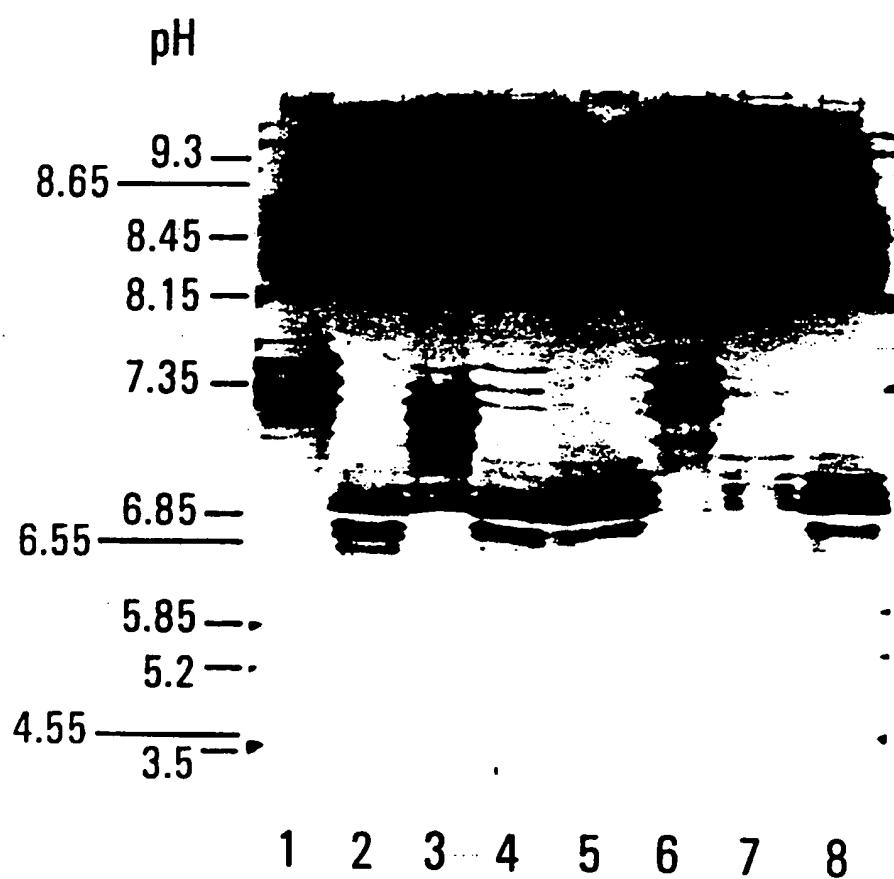


FIG. 5b.



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FIG. 6.



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FIG. 7a.

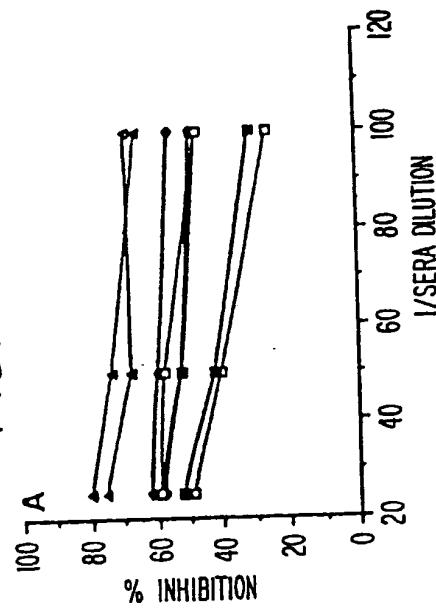


FIG. 7c.

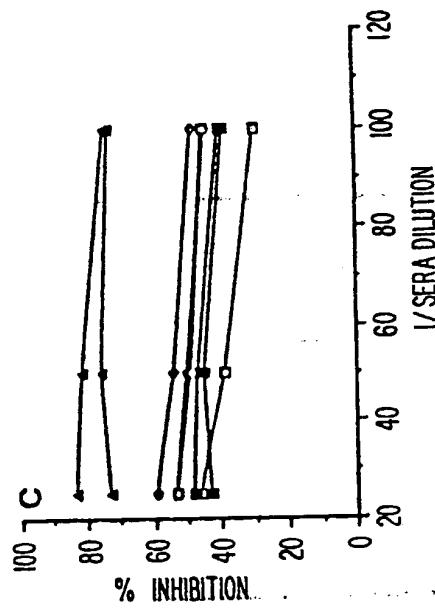


FIG. 7b.

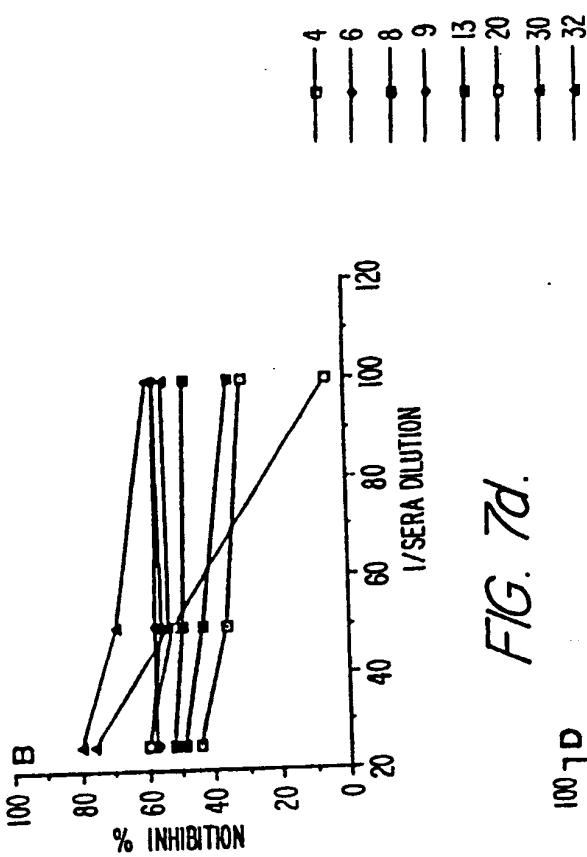
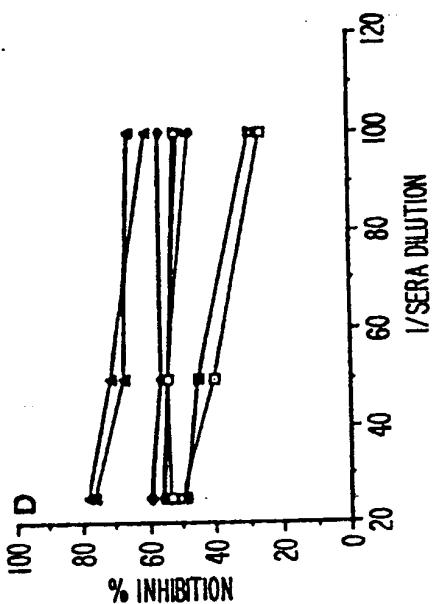


FIG. 7d.



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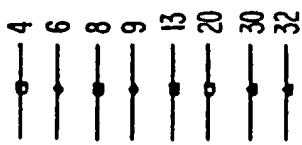


FIG. 7f.

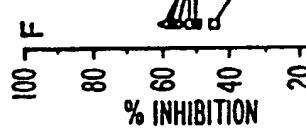


FIG. 7h.

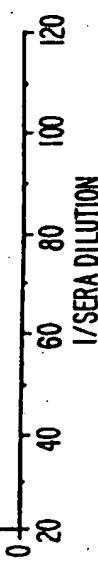
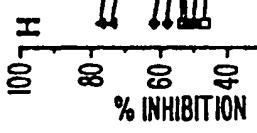


FIG. 7e.

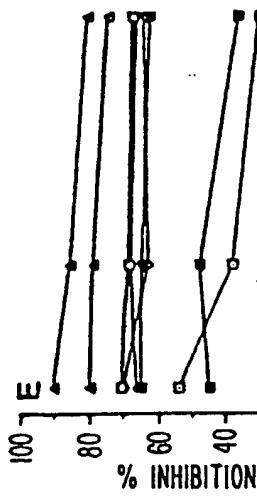
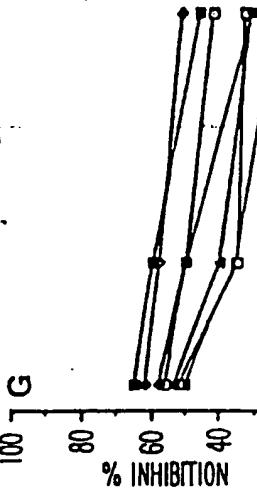


FIG. 7g.



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FIG. 8a.

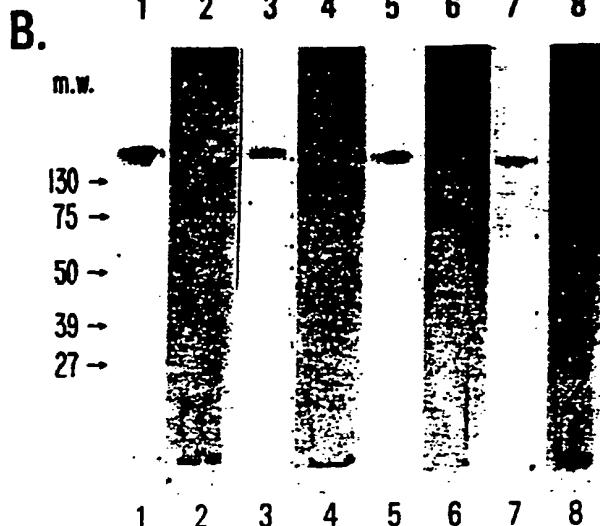
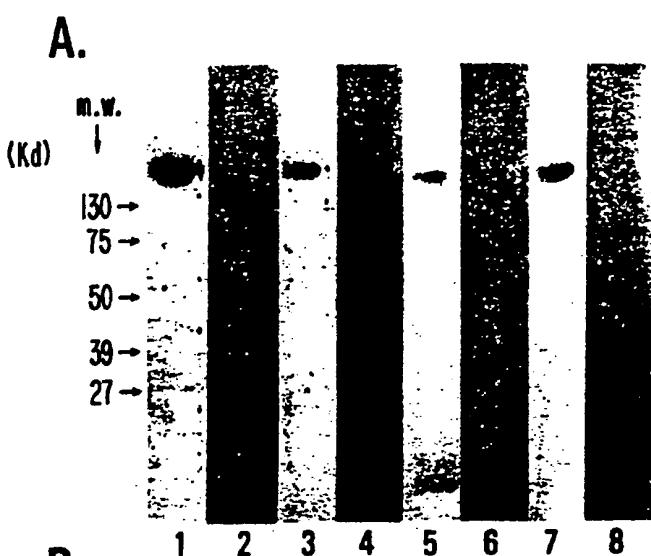


FIG. 8c.

C.

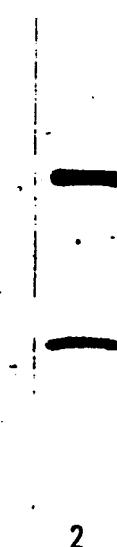


FIG. 8b.

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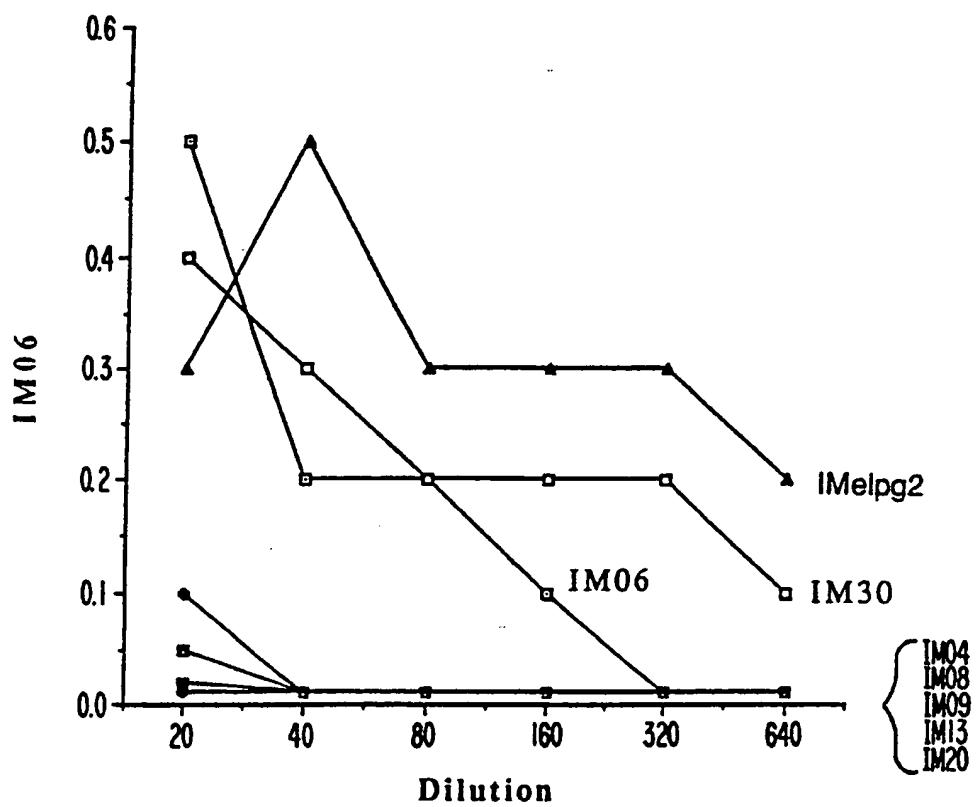


FIG. 9.

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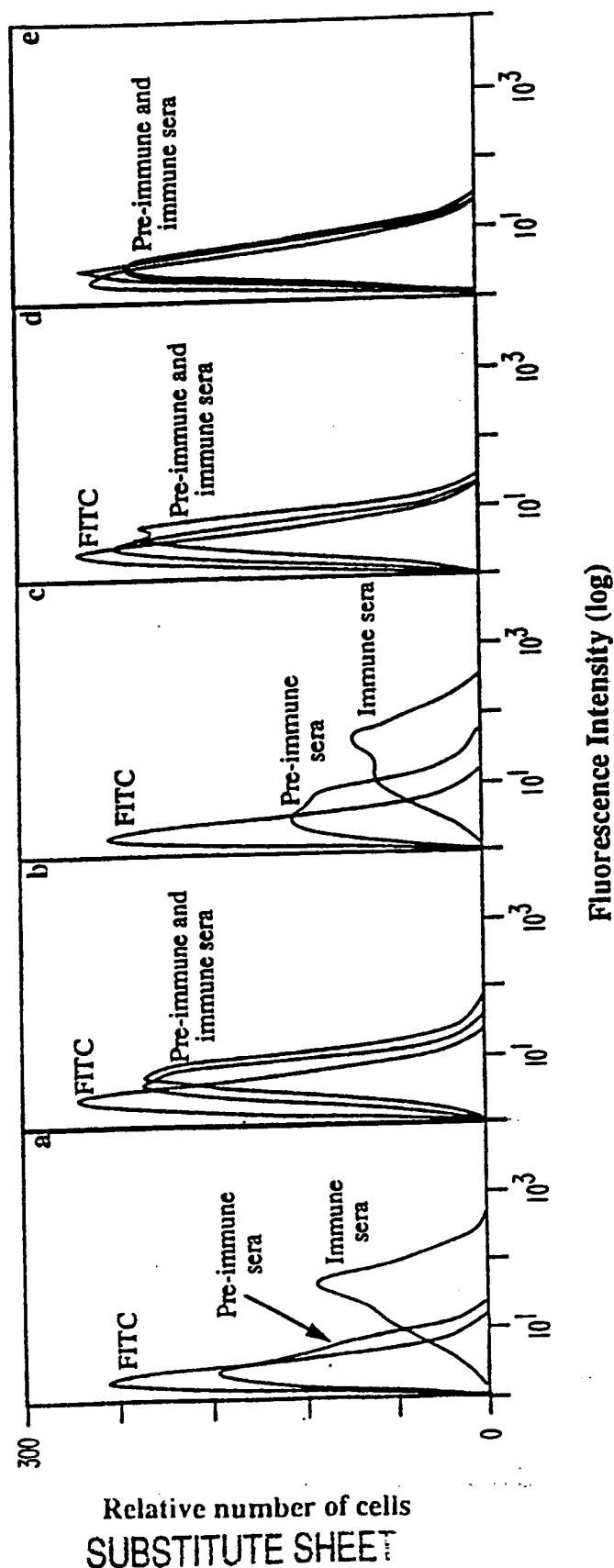


FIG. 10

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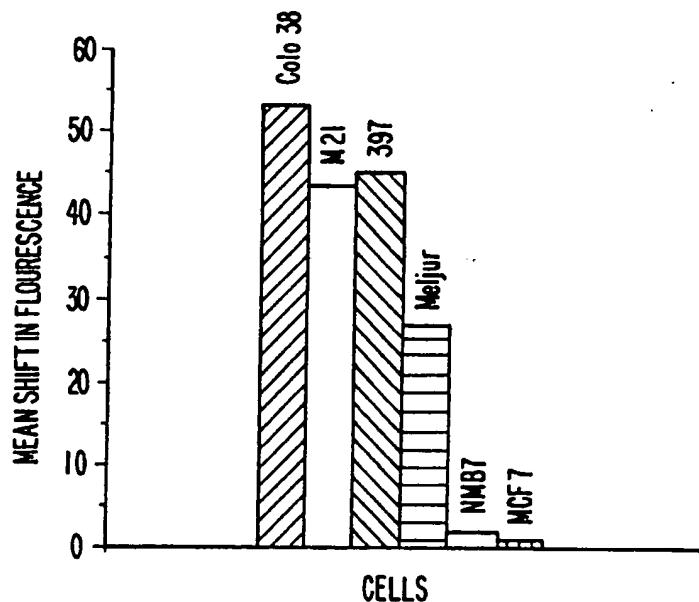
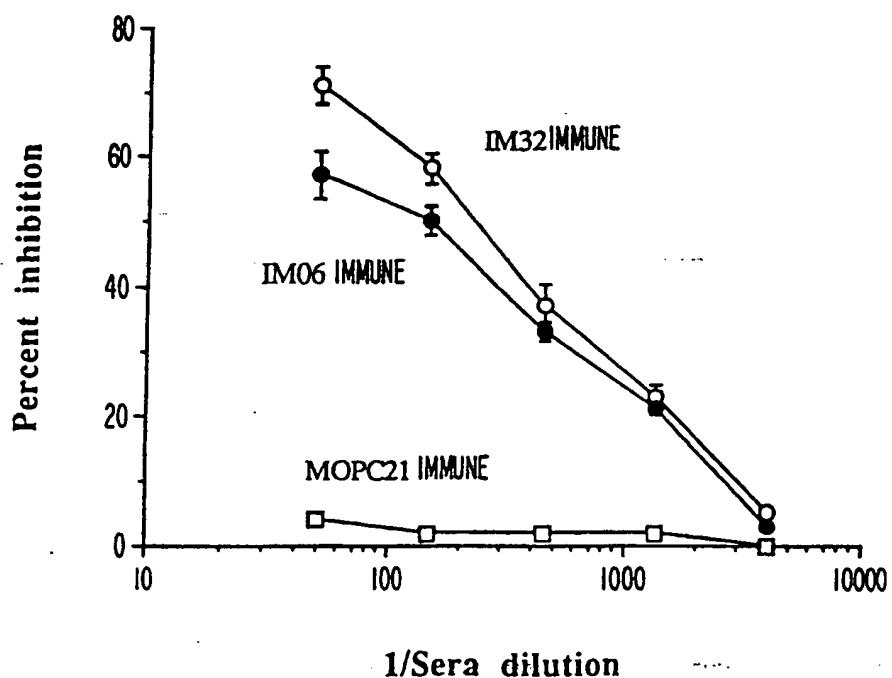


FIG. 11.

FIG. 12.



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FIG. 13.

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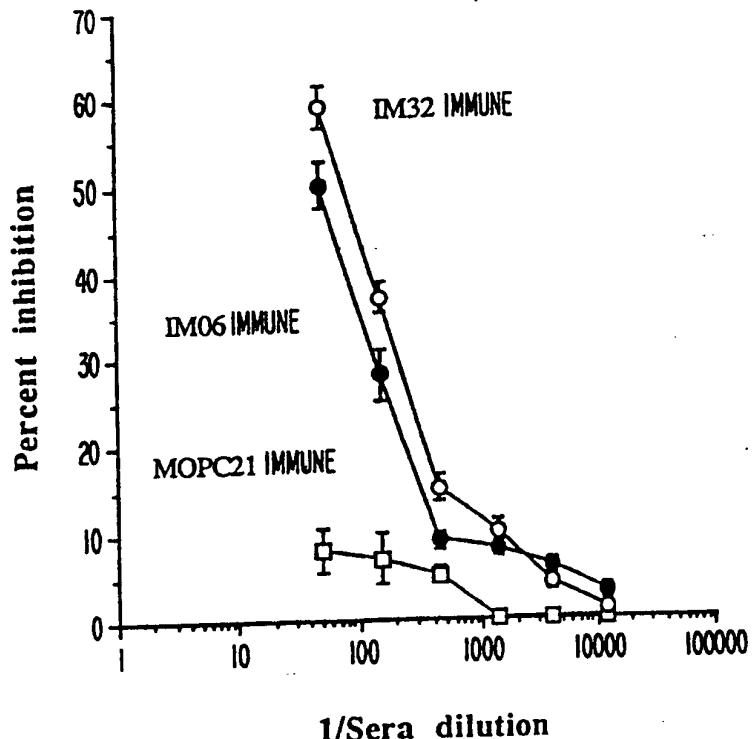
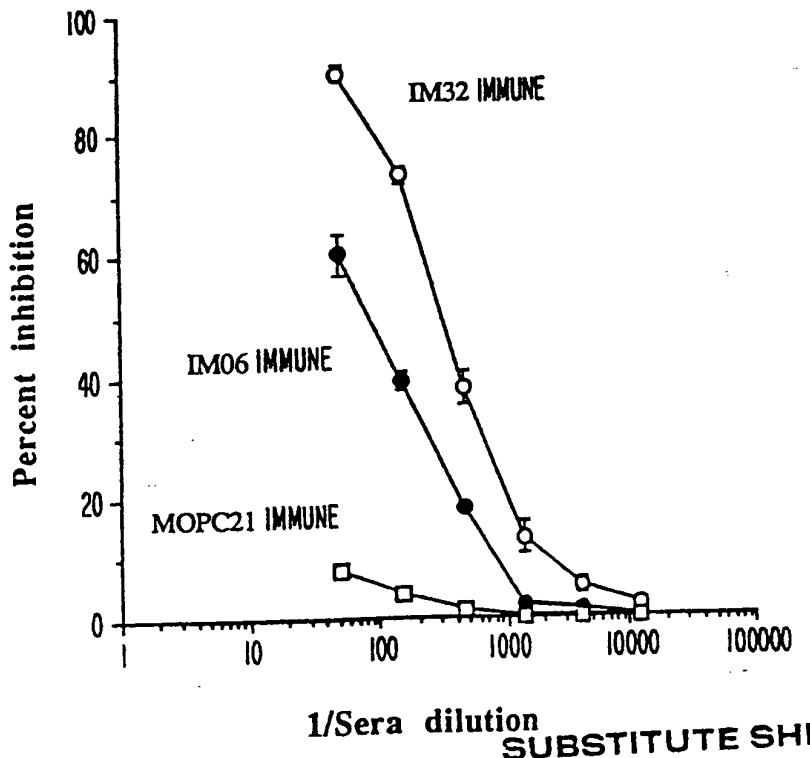
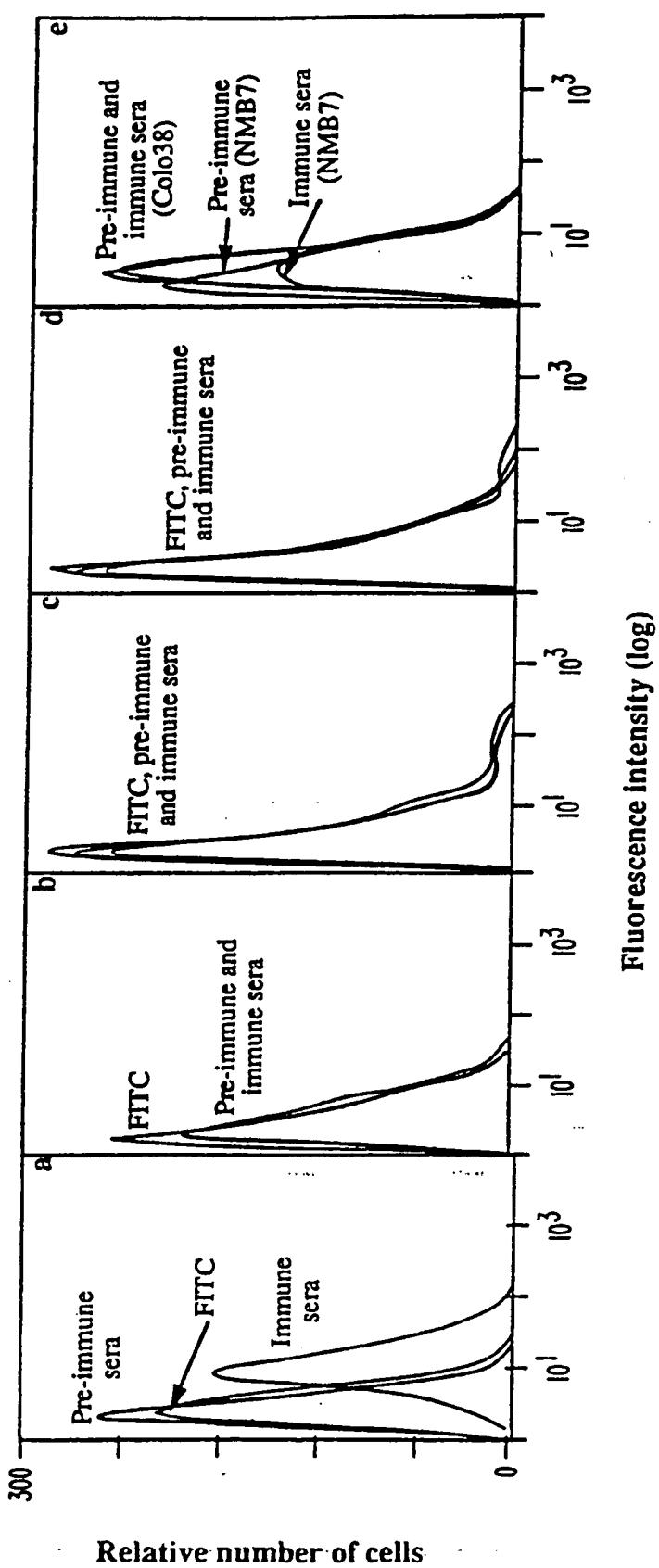


FIG. 14.



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FIG. 15.



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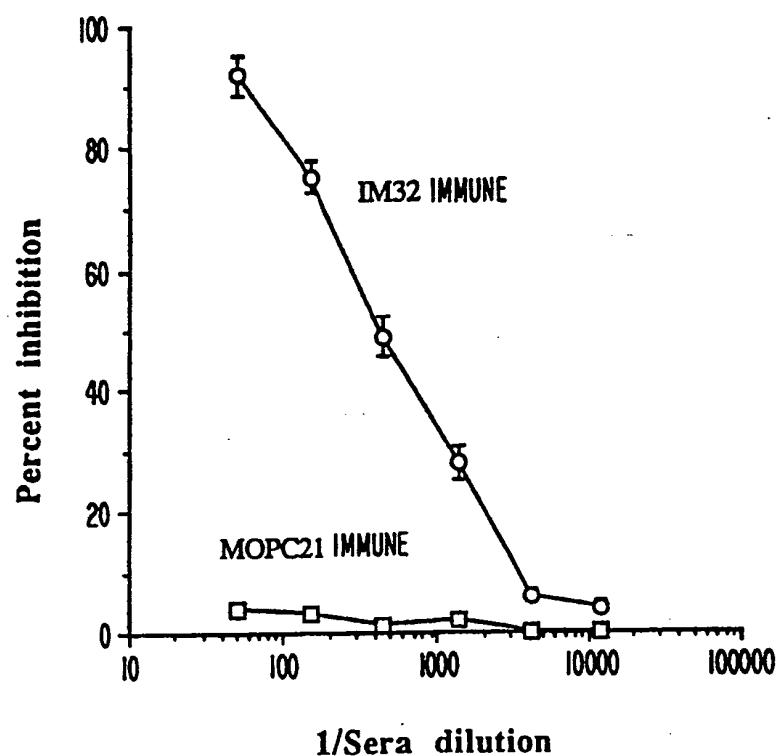
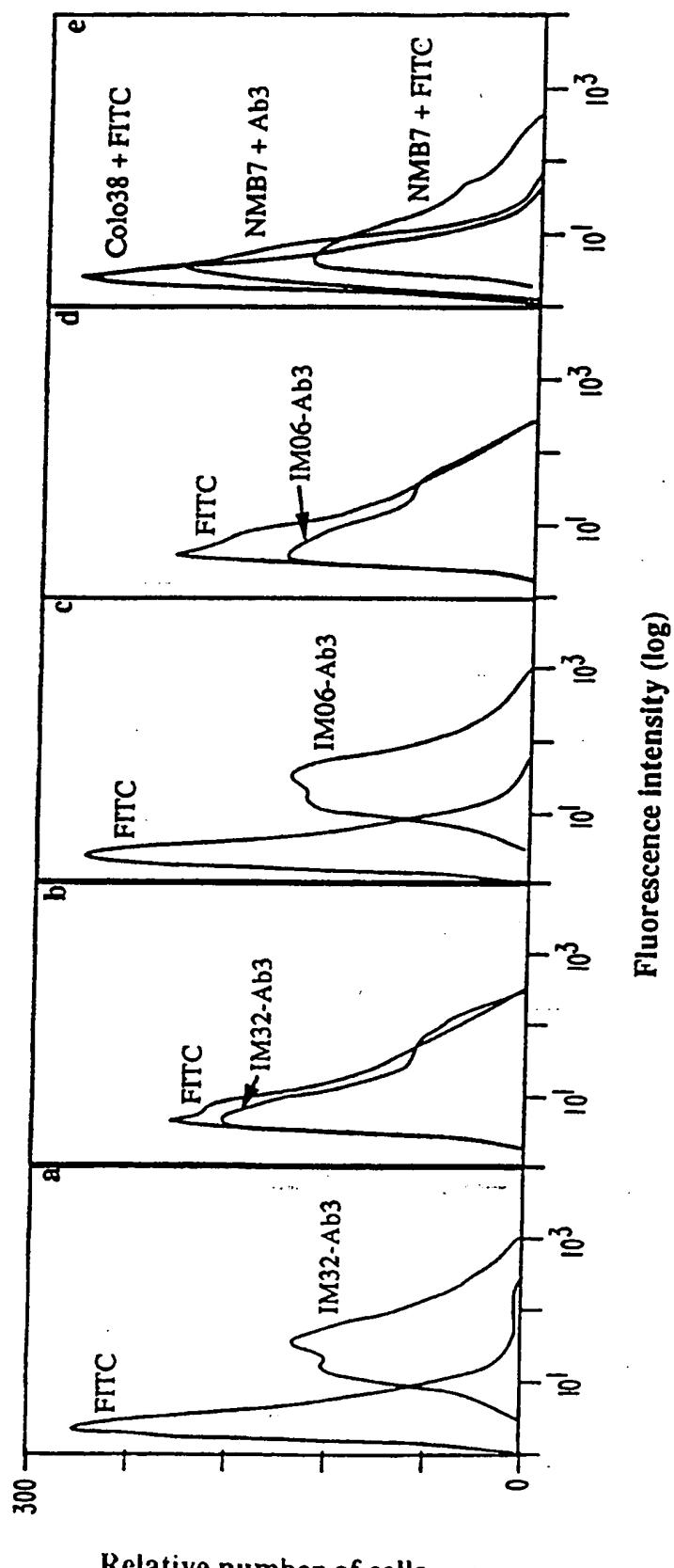


FIG. 16.

FIG. 17.



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FIG. 18a.

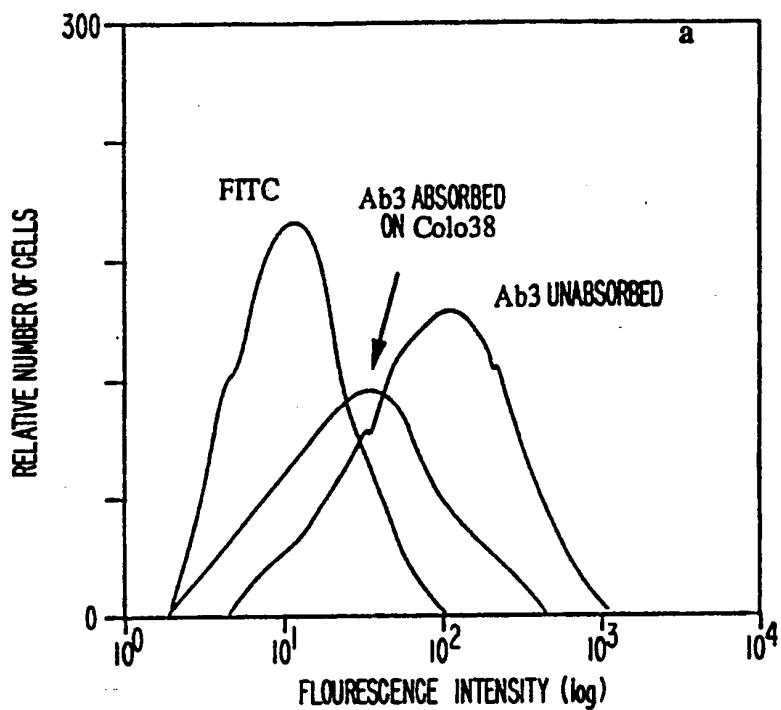
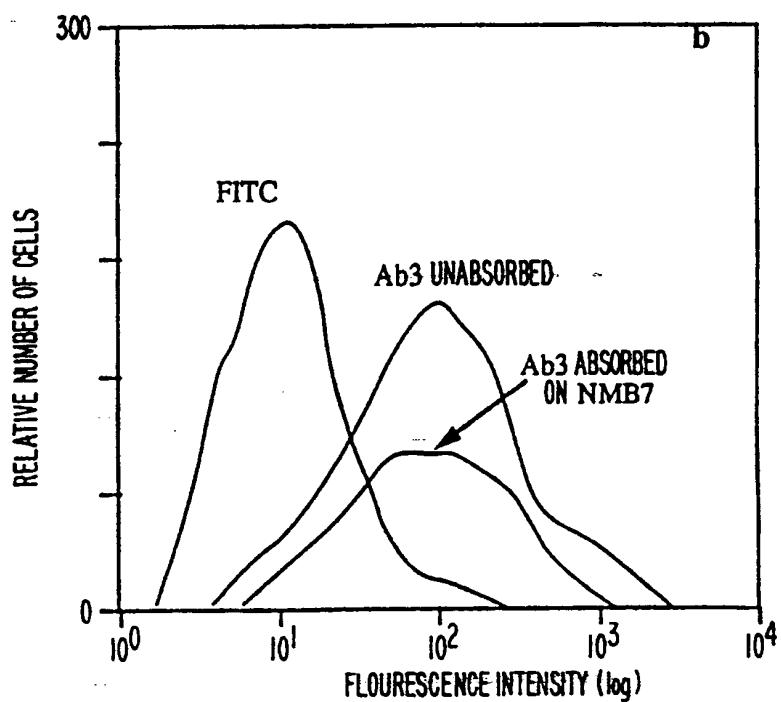


FIG. 18b.



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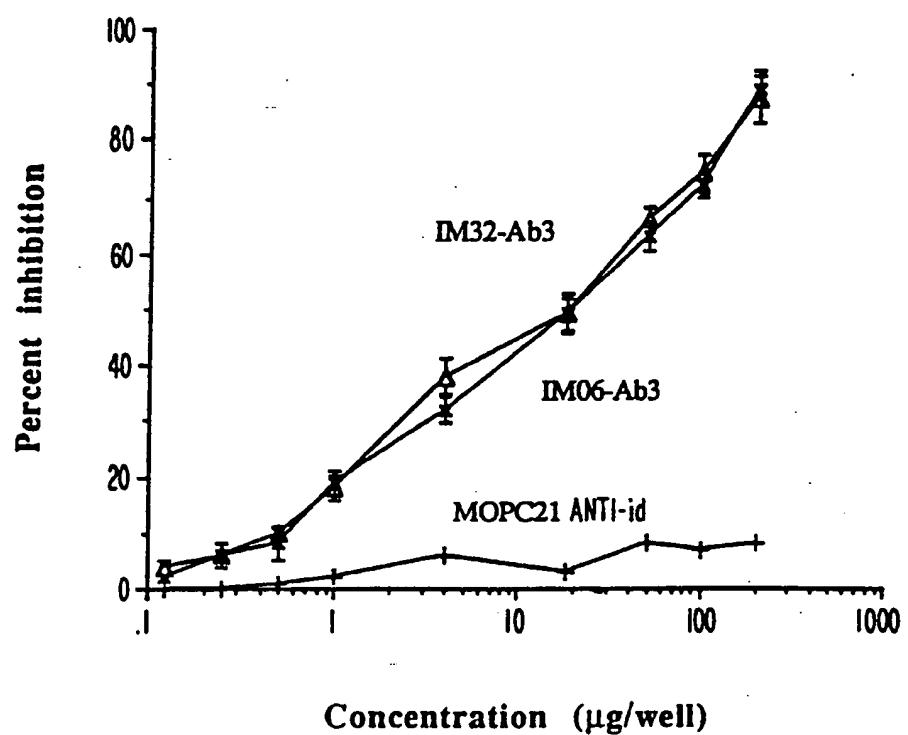


FIG. 19.

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FIG. 20b.

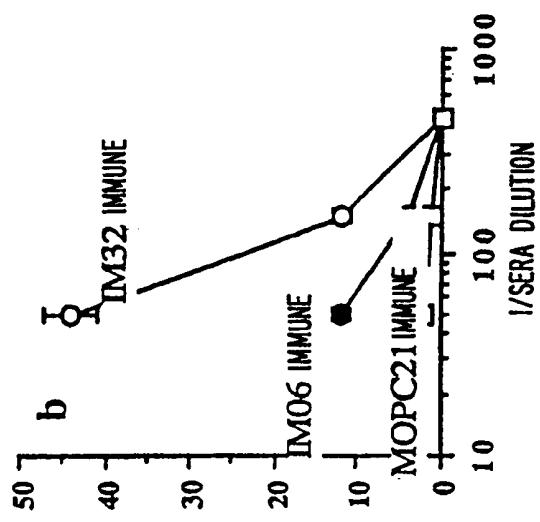
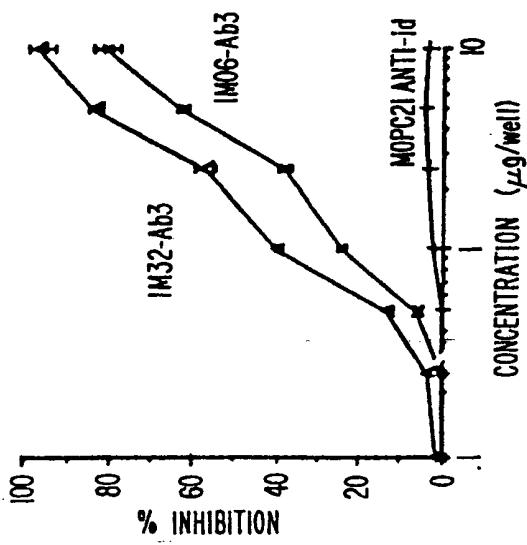


FIG. 20a.



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FIG. 2/a.

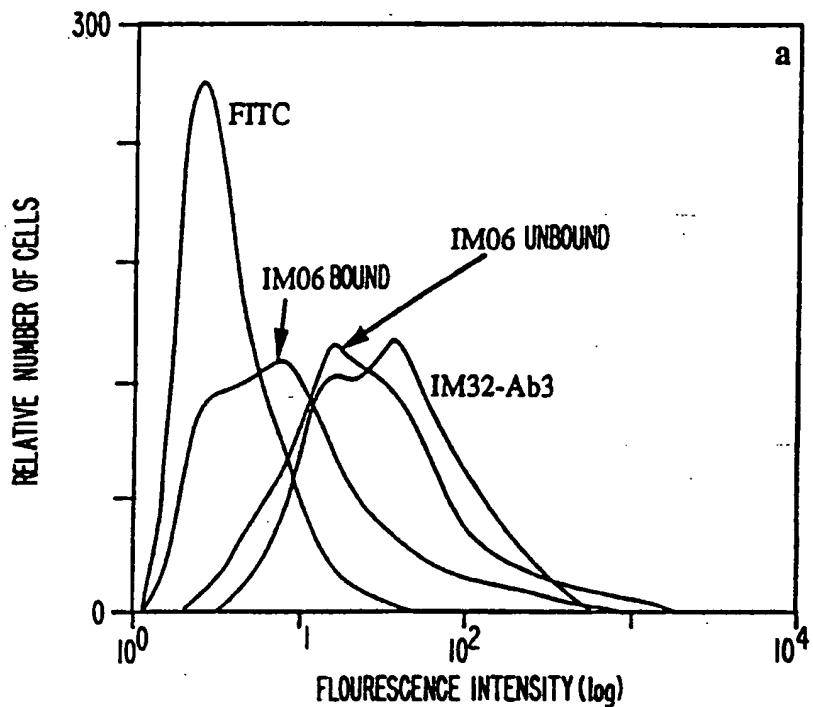
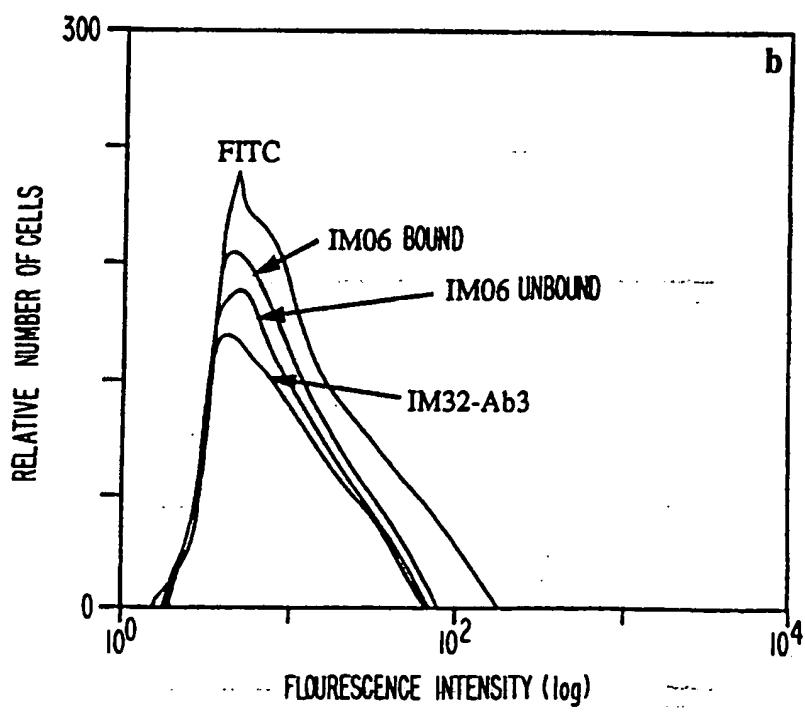


FIG. 2/b.



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FIG. 22.

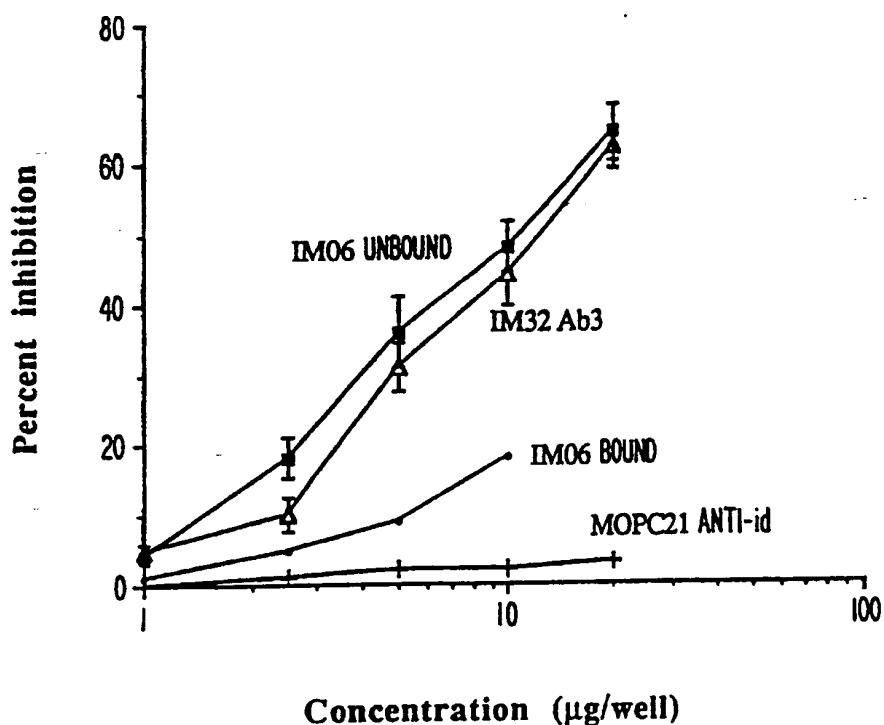
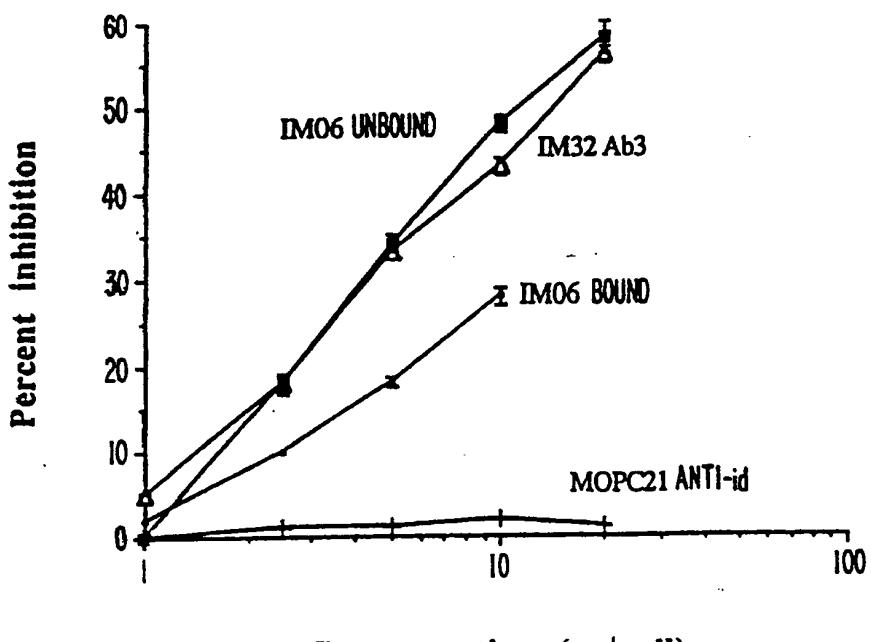


FIG. 23.



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## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 92/01711

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12P21/08; A61K39/395; G01N33/53

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.Cl. 5	C12P ; A61K ; G01N

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO,A,8 911 296 (FERRONE) 30 November 1989 see the whole document ---	1-12
X,P	EP,A,0 428 485 (NEW YORK MEDICAL COLLEGE) 22 May 1991 see the whole document ---	1-12
X	CANCER RES. vol. 47, no. 16, 1987, pages 4312 - 4317; M. KUSAMA: 'Syngeneic antiidiotype antisera' see the whole document ---	1-12
X	J. IMMUNOL. vol. 143, 1989, pages 3844 - 3852; M. KUSAMA: 'Characterisation of syngeneic antiidiotypic monoclonal antibodies' see the whole document ----- -/-	1-12

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- <sup>Z</sup> document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

1 10 JULY 1992

Date of Mailing of this International Search Report

24.07.92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

SKELLY J.M.

J. Skelly

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PROC. AMER. ASSOC. CANCER RES. vol. 31, 1990, page 474; S. FERRONE ET AL.: 'Active specific immunotherapy with murine antiidiotypic monoclonal antibodies' see the whole document -----	1-12

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. US 9201711  
SA 58719

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8911296	30-11-89	AU-A- 3766389 EP-A- 0380607 JP-T- 3501209	12-12-89 08-08-90 22-03-91
EP-A-0428485	22-05-91	CA-A- 2029714 JP-A- 3206894	15-05-91 10-09-91

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